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The Effects of Phytohormones on Growth and Artemisinin Production in Hairy Root Cultures of Artemisia Annua L.

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THE EFFECTS OF PHYTOHORMONES ON GROWTH AND ARTEMISININ PRODUCTION IN HAIRY ROOT CULTURES OF *ARTEMISIA ANNUA* L.

By

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ABSTRACT

The *in vitro* addition of plant growth regulators (i.e. phytohormones) to *Agrobacterium* transformed hairy root cultures affects morphological and biochemical changes, resulting in altered growth and secondary metabolite accumulation rates in root tissues. Significant increases in both growth and secondary product accumulation have been observed, upon incubation with phytohormones, in some species. Consequently, the use of phytohormones *in vitro* has received increasing attention as a potential means for increasing those plant secondary products notoriously produced in small quantities. However, currently little is known about the specific effects of phytohormones on growth and secondary metabolism.

The Chinese herb *Artemisia annua L*. produces artemisinin, an effective antimalarial therapeutic. Efforts to increase the amount of artemisinin via chemical synthesis or field-grown crops have met with huge costs and disappointingly low yields, respectively. *Agrobacterium* transformed hairy root cultures of *A. annua* (Clone YUT16) produce artemisinin and undergo rapid growth compared to non-transformed, making them a good model system to study secondary metabolite production.

Demonstrated herein is the first definitive evidence, by any hairy root species, of a favorable response to exogenous combinatorial hormone application as well as the development of a two-stage culture system alluding to optimal growth and artemisinin production conditions in *A. annua* hairy roots. Furthermore, analysis of artemisinin and biomass accumulation in *A. annua* hairy roots in the presence of phytohormones has revealed effective individual as well as combinatorial phytohormone concentrations suitable for increasing single and bulk root growth, and artemisinin production. The effectiveness of an

optimal phytohormone combination, with respect to time of addition, its relationship to inoculum size, and its combination with the provision of fresh nutrients and or mechanical stress to the roots is also described resulting in artemisinin yields of up to 0.8 μg/g F.W. Although the findings contained herein are not yet optimized they do, however, argue for the potential usefulness of a two-stage production scheme using phytohormones to increase plant secondary metabolite production *in vitro*.

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CHAPTER 1

1.0. Background and General Overview

1.1.1. Terpenoids: Valuable Secondary Metabolites

Secondary metabolites are those compounds that are produced exclusive of the primary (nutrition and maintenance) metabolites essential to sustain the life of an organism. These secondary products have been shown to be very useful for both the plants as well as the animals that synthesize them (Facchini, 1999). The most abundant and structurally dissimilar group of secondary metabolites is the terpenoid family (or isoprenoids). Terpenoids are a class of secondary metabolites, derived from isopentenyl diphosphate (IPP), with nearly 22,000 members exhibiting more than 300 ring systems (van der Hoevena *et al.* 2000). Terpenoids, including the retinoids, the geranylgeranyl and farnesyl protein anchors, vitamins A, D, and E, coenzyme Q, cholesterol, and the steroid hormones have been known to play decisive roles in organisms. Plants direct growth and development via regulatory terpenes that include the gibberellins, the brassinosteroids, and abscisic acid. Some of these compounds are medicinally important, for instance, ginkgolide, which has been shown to delay the inception and progression of Alzheimer's disease (van der Hoevena *et al.* 2000) as well as taxol, used as an anticancer agent (van der Hoevena *et al.* 2000). Production of certain protective terpenoids, by various plant species, has also been shown to impair the biological processes in herbivores. Numerous plant terpenoids have been found to perform secondary functions, primarily in defense against insect herbivores (van der Hoevena *et al.* 2000). Certainly terpenoid-based resins, produced by conifers, have long been studied for their industrial importance and role in defense against herbivores and pathogens (Bohlmann

and Croteau, 1999; Bohlmann et al. 2000; Phillips and Croteau, 1999; Trapp and Croteau, 2001).

1.1.2. Pathways Governing Terpenoid Biosynthesis

Since the 1950's, it was known that the central components for terpenoid biosynthesis, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are synthesized from activated acetic acid via the mevalonic acid pathway (Eisenreich *et al.* 1998). Today there is a wealth of evidence that shows that an additional pathway exists for the formation of IPP and subsequent terpenoid synthesis in higher plants. The mevalonate pathway is located in the cytosol/ endoplasmic reticulum, and the 2-C-methyl-erythritol-4-phosphate pathway (mevalonic acid-autonomous or MEP), which proceeds via 1-deoxyxylulose-5-phosphate, is localized in plastids (Eisenreich *et al.* 1998; Lichtenthaler, 1999; Figure 1.1). In our laboratory, Souret (2002) provided the first evidence of these dual pathways in *Agrobacterium* transformed hairy roots of *Artemisia annua.* By RNA gel blot analysis, he showed constitutive expression of key regulatory enzymes in the MEP pathway (Souret, 2002; Souret *et al*. 2002). In general, however, regulation of terpenoid synthesis is very poorly understood (Crouteau *et al.* 2000).

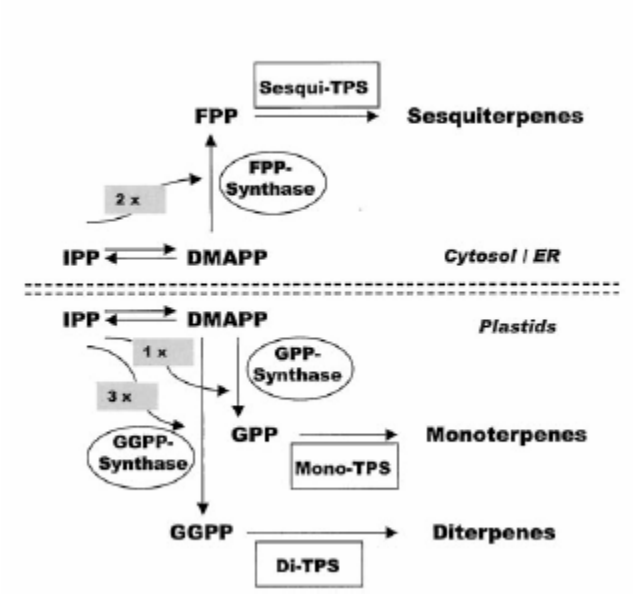

Figure 1.1. Scheme of the pathways of terpenoid biosynthesis taken from Martin *et al*. (2002).

1.1.3. Artemisinin: An Effective Antimalarial Sesquiterpene

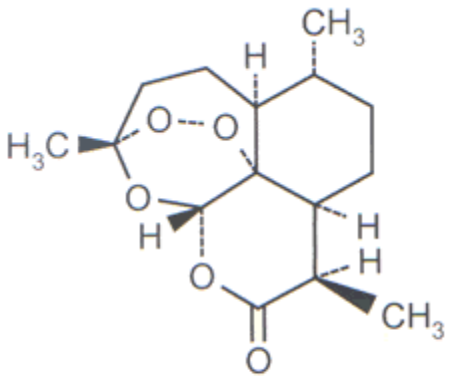

Figure 1.2. Structure of artemisinin.

Structurally, sesquiterpenes can be acyclic (farnesol), monocyclic (abscisic acid), or polycyclic (artemisinin). Many also exhibit biological activity, for example, the plant growth regulator abscisic acid (ABA) (Threlfall and Whitehead, 1991). Our laboratory focuses on terpenoid biosynthesis, specifically the production of the sesquiterpene lactone, artemisinin, that has potent biological activity (Figure 1.2). It and its derivatives are effective anti-malarial therapeutics, produced by the annual herb, *Artemisia annua L., (Asteraceae)*, native to Asia, (Dhingra *et al*, 2000), and known in the United States as Sweet Annie, or annual wormwood. Artemisinin was isolated and correctly defined in 1972 in China as a sesquiterpene lactone with an endoperoxide bridge. This peroxide moiety is responsible for its anti-parasitic activity in the treatment of malaria (Dhingra *et al*, 2000; Duke *et al*. 1987; Chen *et al*. 1991). Artemisinin is now commercially available as a therapeutic against drug-resistant strains of Plasmodium, the malarial parasite (Duke *et al*. 1987; Chen *et al*. 1991). Unfortunately artemisinin production by the *A. annua* plant is usually in the range of 0.01% to 0.4%, but some clones can produce over 1% of the plant's dry weight (Delabays *et al*. 1993; Delabays *et al*. 2001). Acton *et al*. (1985), reported that artemisinin can also be obtained from artemisinic acid, which can be found at concentrations as much as 10-fold higher than artemisinin in hairy root tissues (Weathers unpublished results), and the native plant (Ferreira *et al.* 1997).

1.1.4. Artemisinin Optimization

In *Artemisia annua,* the maximum artemisinin accumulation, from field grown crops, takes place at or near the onset of flowering in most strains, hence only one crop can be harvested annually (Woerdenbag *et al*. 1994; Morales *et al*. 1993; Ferreira *et al*. 1995; Laughlin 1995). Also, the highest concentrations obtained at harvest are 0.8% and have been reported to be as low as 0.001% of the plant's dry weight (Martinez and Staba, 1988). This is equivalent to 8 mg/g and 0.01mg/g dry weight, respectively (Basile *et al.* 1993). Klayman (1985) reported that in clinical trials, one course of treatment requires 2-3 grams of the drug and that repeated recrudescence may call for multiple courses of treatment per year. Since malaria threatens roughly 40 % of the world's population causing death, morbidity, and significant socio-economic loss, a considerable amount of artemisinin is required (Dhingra *et al*, 2000). The limited availability from large-scale cropping of *A. annua* plants coupled with high demand for the therapeutic has spurred scientists to explore alternate methods of production. Chemical synthesis of artemisinin has been attempted. However, due to the presence of the endoperoxide moiety in the structure of artemisinin, present methods for chemical synthesis are expensive and have met with limited success (Dhingra *et al,* 2000).

An alternative to field-grown crops or to chemical synthesis has been large scale *in vitro* plant tissue culture (Francois *et al.* 1990; Dhingra *et al*, 2000). *In vitro* cultures may potentially constitute useful and easily manipulated systems for producing valuable biologically active compound in plants that do not require labor-intensive methods (McCabe *et al*. 1997). However, *in vitro* production systems are still not cost effective and produce low yields (McCabe *et al*. 1997).

1.1.5. *In Vitro* Methods for Overproduction of Secondary Metabolites

1.1.5.1. Undifferentiated vs. Differentiated Culture

Alternative methods such as cell suspension cultures have been sought to deal with problems of low concentrations in whole plants of secondary metabolites, like artemisinin (Basile *et al*. 1993) and paclitaxel (Christen *et al*. 1991; Gibson *et al*. 1995; Luo *et al,* 2001). While suspension cultures can be transformed with novel genes and are relatively easy to scale-up, they are not very genetically stable (Bais *et al*, 2001;Gibson *et al*. 1995; Rhodes *et al.* 1994). Disappointing yields coupled with instability have required new methods for improving production of secondary products.

Toward this end, genetic manipulation of dicotyledonous whole plants using modified Ri or Ti DNA segments from agropine- and mannopine-type strains of *Agrobacterium*, has resulted in neoplastic roots that have augmented growth and secondary metabolite production rates (Bais *et al.* 2001; Giri and Narasu, 2000). Secondary product levels in *in vitro* differentiated tissues, are frequently either comparable or greater than those found in either roots or shoots of the intact plant (Giri and Narasu, 2000; Dhingra *et al*, 2000; Bais *et al.* 2001; Bourgard *et al.* 2001). These compounds are also produced concomitantly with growth (Bourgard *et al.* 2001). Therefore, continuous extraction of secondary metabolites from actively growing hairy roots is possible. Moreover, the transformants are genetically stable over a long period of time with consistent biosynthetic capabilities. Also, hairy roots do not require the application of growth regulators. (Yu and Doran, 1994; Bias *et al.* 2001). In 1994, Weathers *et al*. reported high levels (0.4% of the dry weight) of artemisinin in hairy root cultures of *A. annua*.

While one cannot preclude the value of suspension cultures, their inherent genetic instability dilutes their potential as feasible secondary metabolite production systems. Conversely, the long term genetic stability, and consistent biosynthetic capabilities of transformed hairy roots predisposes these differentiated cultures as a better means for secondary metabolite production *in vitro*.

1.1.5.2. Abiotic Factors Affecting Secondary Metabolite Production in Hairy Roots

Many factors including light, medium composition, pH and most notably, the exogenous application of plant growth regulators affect secondary metabolism in *in vitro* hairy root cultures. The relative impact of these influences is usually species dependent (Bourgaud *et al.* 2001).

Researchers studying alkaloid production in transformed hairy root cultures noted that optimization of the medium, specifically mineral composition and sucrose concentration can play either a neutral or positive role in growth of the roots as well as secondary metabolite production (Hilton and Rhodes 1993; Rhodes *et al.* 1994; Bourgard *et al.* 2001). The external pH of the medium changes over the course of batch growth, and has also been shown to influence the release of secondary products from root cultures (Sáenz-Carbonell *et al*, 1993; Morgan *et al.* 2000).

Of interest to this study is the relationship between exogenously applied phytohormones and hairy root cultures, specifically their effects on root development and secondary metabolism. Endogenously produced in a variety of plant tissues, plant hormones are present in trace amounts and are responsible for an array of developmental processes that involve abiotic as well as biotic factors (Crozier *et al.* 2000). Since the discovery of these

signaling compounds in the mid 1920's, a considerable amount of research has been performed examining the influences of both endogenous and exogenous concentrations of phytohormones on plant cell physiology. Currently, we know that these growth regulators direct culture growth, influence cell secondary metabolite accumulation, and promote morphological diversity in a concentration dependent manner (Crozier *et al.* 2000). There are five main phytohormones: gibberellins, auxins, cytokinins, ethylene, and abscisic acid. Following is a short review of the 5 main plant hormones, along with a summary of their main and observed affects on hairy roots.

1.1.6. Phytohormone Regulation of Growth and Secondary Metabolites

1.1.6.1. Gibberellic Acid (GA)

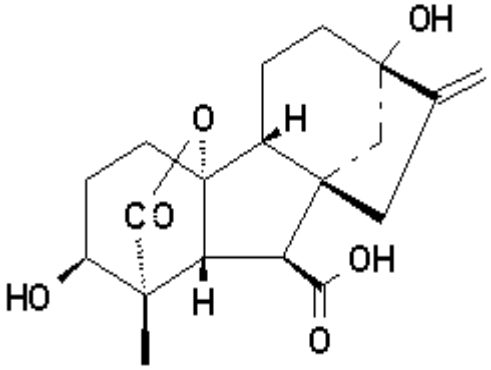

Figure 1.3. Structure of Gibberellic acid from Crozier *et al*. (2000).

Gibberellic acids (GA) are widespread and so far ubiquitous in angiosperms and gymnosperms as well as pteridophytes (Crozier *et al.* 2000; Figure 1.3). They have also been isolated from lower plant species such as mosses and algae, at least two fungal species, and from two bacterial species (Crozier *et al*, 2000). Since their discovery in the ascomycete, *Gibberlla fujikuroi* in 1926, a phenomenal number of GAs (125) have been characterized to date, all of which are most likely not essential to the plant (Crozier *et al*, 2000). GAs are products of the terpenoid pathway (Crozier *et al*, 2000). Following are some of the responses known for gibberellins:

- Stimulate stem elongation by stimulating cell division and elongation.
- Stimulate bolting/flowering in reaction to long days.
- Break seed dormancy in some plants, which require stratification or light to induce germination.
- Induce male character in dioecious flowers (sex expression).
- Stimulate enzyme production (α-amylase) in germinating cereal grains for recruitment of seed reserves.
- Can initiate seedless fruit development.
- Can delay senescence in leaves and citrus fruits.

1.1.6.1.2. GA Affects on Hairy Root and Secondary Metabolism

Fundamental to hairy root culture systems, is their ability to grow without the addition of plant growth regulators. Interestingly, the addition of an individual growth regulator, like gibberellic acid, has yielded definitive evidence that GA can play an important physiological role in root morphology and secondary product accumulation.

In studies of the effects of GA on hairy root morphology, Bais *et al.* (2001) reported that low concentrations (0.5 mg/l) of GA increased the biomass accumulation in the hairy roots of *Cichorum intybus,* due to high levels of root elongation, lateral root branching, and primary root growth almost 2-fold over the hormone-free controls. Vanhala *et al.* (1998) observed, in the presence of GA no loss of *Hyocyamus muticus* root integrity but that the hairy root cultures seemed to age rapidly compared to roots not supplied with GA. Surprisingly, this premature aging phenomenon did not decrease biomass accumulation. Ohkawa and coworkers (1989) observed that GA stimulated elongation and lateral branching of the hairy roots of *Datura innoxia.* Their roots were transformed with TL-DNA (the portion of Ti DNA lacking the IAA synthesizing genes) and displayed rapid growth and

copious amounts of lateral roots in the presence of GA at concentrations between 0.01-1.0 mg per liter. It was concluded that GA, rather than auxin, was responsible for controlling root morphology in *D. innoxia*. Also, exogenously supplied GA_3 enhanced branching in *Brugmansia candida* roots (Rhodes *et al.* 1994). A pronounced influence on growth was observed by Liu *et al.* (1997) in the hairy roots of *A. annua* in the presence of GA at a concentration of 5 mg/l. Of all the hormones studied in that report, GA was the only one to yield high levels of biomass. This may be a reflection of the increase in root elongation and lateral branching as seen in other studies involving hairy root cultures. For example, in our laboratory Smith *et al*. (1997) observed that GA_3 concentrations of 1-10 ng per liter provided the most significant increase in biomass during exponential growth in *A. annua* hairy roots.

In terms of secondary metabolite production, variable results have been obtained using GA, and are usually species specific. For example, Vanhala *et al.* (1998) observed that in *H. muticus* hairy roots, GA decreased the accumulation of the secondary metabolite hyoscyamine, compared to roots cultivated in hormone-free medium. Conversely, Liu *et al* (1997) and Bais *et al* (2001) observed that the addition of GA to the growth medium increased artemisinin and coumarin content in the hairy roots of *A. annua* and *C. intybus* L. CV. Lucknow local, respectively. Since GA is known to induce flowering in some plants (Crozier *et al.* 2000), and maximum artemisinin accumulation in field-grown crops occurs at or near the onset of flowering in most strains (Woerdenbag *et al*. 1994; Morales *et al.* 1993; Ferreira *et al*. 1995; Laughlin 1995), this may explain the increase in artemisinin. In the case of coumarin production, Bais *et al*. (2001) observed that coumarin accumulation was strictly related to growth, suggesting that GA plays a key role in the production to coumarin. From

our laboratory, Smith *et al.* (1997) reported that GA_3 concentration of 10 ng per liter produced the highest levels of artemisinin in hairy roots of *A. annua.*

1.1.6.2. Auxins

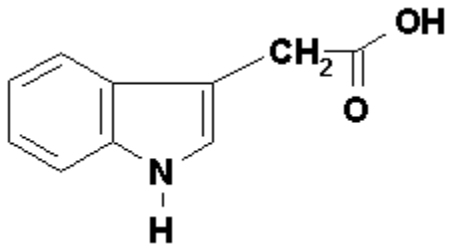

Figure 1.4. Structure of auxin, taken from Crozier *et al.* (2000).

Another growth-promoting factor, auxin (Figure 1.4), was discovered in 1926, by Frits Went (Crozier, *et al.* 2000). Indole-3-acetic acid (IAA) represents the primary auxin produced in plants. Auxin is a diffusible compound that is chemically similar to the amino acid tryptophan, from which auxins are thought to be synthesized (Crozier *et al.* 2000). Following are some of the responses known for auxins:

- Stimulate root initiation on stem cuttings and lateral root development in tissue culture.
- Mediate the tropistic response of bending in response to gravity and light.
- Suppress the growth of lateral buds.
- Delay leaf senescence.
- Can hinder or promote (via ethylene stimulation) leaf and fruit abscission.
- Promote (via ethylene production) femaleness in dioecious flowers.
- Can direct fruit location and growth in some plants.
- Are involved in movement toward auxin possibly by an effect on phloem transport.
- Promote in Bromeliad flowering.
- Postpone fruit ripening.
- Stimulate growth of flower parts.

- Stimulate the production of ethylene at high concentrations.

1.1.6.2.1. Auxin Affects on Hairy Root Secondary Metabolism

The mannopine or agropine-type strains of *A. rhizogenes* used to transform plant tissues, impart auxin-sensitive characteristics to the resulting transformants. Physiologically, this character is manifested either via a heightened sensitivity to auxin, or by a hypersensitivity to auxin along with elevated levels of IAA inside the tissues, respectively (Giri and Narasu, 2000; Arroo *et al.* 1995). Hairy root auxin sensitivity has received great attention. Moreover, studies on the biochemical relationship between exogenous and endogenous auxin levels using these differentiated tissues have returned interesting findings in the area of valuable root-derived biologically active compounds. By and large, researchers investigating the physiological role of exogenously applied auxin in root growth and secondary metabolite production have established that these signal molecules can greatly impact plant tissue stability and secondary product accumulation either individually or through phytohormone-phytohormone interactions.

Of particular interest to this study is the effect of auxin concentration relative to root biomass. For example, Sauerwein *et al.* (1992) reported on the exogenous phytohormone-phytohormone relationship displayed in cultures of *Hyocyamus albus* and described the formation of pure calli in the presence of both exogenous auxin and cytokinin. They observed that the single addition of only IAA to the medium resulted in partial disorganization of the hairy root morphology. However, growth rates increased, depending upon the transformation strain used, when there were low levels of auxin and cytokinin (1:1mg/l ratio). Rhodes *et al.* (1994) also observed disorganization of the hairy root matrix of *Nicotiana rustica* when cultures were supplied with auxins and cytokinins. However,

these effects were minimized by increasing the root inoculum size. Bais *et al.* (2001) also showed that when high levels of exogenously applied auxins, specifically IAA and NAA, were added in the presence of low exogenous cytokinin levels, there was a decrease in biomass in the hairy root cultures of *C. intybus*, unless the root inoculum size was increased. Although others have shown that auxin addition to a variety of hairy root cultures stimulates growth, the response is likely species specific (Arroo *et al.* 1995; Sudha *et al.* 2003; Luczkiewicz *et al.* 2002). For example, Lin *et al.* (2003) demonstrated that auxin had little influence on hairy root cultures of *Linum flavum*. Taken together, these studies on the effect of auxins on hairy root growth show that, although there is some species variability, the ratio of the exogenously added hormone to root biomass is critical to achieving good root growth.

In terms of auxin effects on secondary metabolite production, Bais *et al.* (2001) noted that high levels of exogenous auxins, specifically IAA and NAA in the presence of low cytokinin levels, decrease the ability of root cultures of *C. intybus* to produce coumarin. Lin *et al*.(2003) showed that coniferin content in *L. flavum* was significantly increased in the presence of auxin. However, in *Tagetes patula* hairy roots, Arroo *et al.* (1995) showed that the addition of IAA inhibited secondary metabolite accumulation. In contrast, addition of either IBA or NAA stimulated ajmalicine and ajmaline production over the *Rauwolfia micrantha* hairy root cultures in hormone-free medium (Sudha *et al.* 2003), whereas Rhodes *et al.* (1994) observed a decrease in nicotine content in the hairy roots of *Nicotiana rustica* when roots were supplied with auxins plus cytokinins. Luczkiewicz *et al.* (2002) discovered that the production of the sesquiterpene lactone pulchelin E was enhanced in hairy roots of *Rudbeckia hirta* compared to that of callus and suspensions cultures in the presence of auxin. In our lab Bunk (1997) suggested that in the presence of high levels of auxin (IAA or NAA),

artemisinic compound yield was increased in *A. annua* hairy roots. Again, these reports show species specificity in auxin's effect on secondary production in hairy roots.

1.1.6.3. Cytokinins

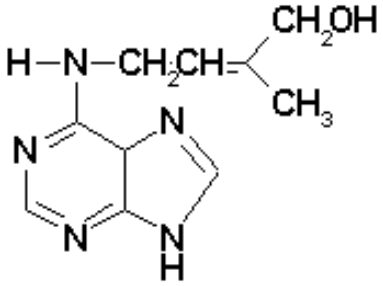

Figure 1.5. Structure of cytokinin, taken from Crozier *et al.* (2000).

The most common form of naturally occurring cytokinin in plants today is called zeatin. Cytokinins (Figure 1.5) have been found in almost all higher plants as well as mosses, fungi, bacteria, and is also associated with the tRNA of many prokaryotes and eukaryotes (Crozier *et al.* 2000). Currently there are more than 200 natural and synthetic cytokinins combined (Crozier *et al.* 2000). Cytokinin concentrations are highest in meristematic regions and areas of continuous growth potential such as roots, young leaves, developing fruits, and seeds (Crozier *et al.* 2000). They are mainly synthesized in the roots and translocated via the xylem to shoots. Cytokinin biosynthesis occurs via the biochemical modification of adenine (Crozier *et al.* 2000). Following are some of the responses known for cytokinins (Crozier *et al.* 2000):

- Stimulate cell division.
- Stimulate morphogenesis (shoot initiation/bud formation) in tissue culture.
- Stimulate the growth of lateral buds-release of apical dominance.
- Stimulate leaf expansion resulting from cell enlargement.
- May enhance stomatal opening in some species.
- Promote the conversion of etioplasts into chloroplasts via stimulation of chlorophyll synthesis.

1.1.6.3.1. Cytokinin Affects on Hairy Root Secondary Metabolism

Cytokinins have been shown to direct shoot growth in tissue culture (Crozier *et al.* 2000). The intrinsic ability of cytokinins to alter tissue morphogenesis favoring the production of shoots may be the reason it has routinely received such little attention in hairy root cultures. In any case, exogenous cytokinins impact growth and secondary metabolite accumulation in a phytohormone-to-phytohormone ratio-dependent manner that can be toxic and even lethal. Likewise, they can induce spontaneous shoot formation in hairy root cultures when administered individually at high concentrations. Indeed, low cytokinin to auxin levels have been shown to induce rapid disorganization in hairy root cultures of *C. intybus* as well as decrease root growth and the ability of root cultures of *C. intybu* L. cv. lucknow local (Bias *et al*, 2001), and *B. candida* (Rhodes *et al.* 1994) to produce secondary products. Vanhala *et al*. (1998) similarly demonstrated that the root growth rate and secondary product accumulation did not change despite addition of exogenous cytokinins to *H. muticus* cultures. In contrast, Sauerwein *et al.* (1992) observed that when the ratio of exogenous cytokinin to exogenous auxin was 1:1, both growth and alkaloid accumulation were enhanced in hairy root cultures of *H. albus*.

In *A. annua*, addition of cytokinin (BAP) to the medium of hairy roots resulted in the disorganization of the root matrix with little effect on production of artemisinic compounds (Bunk, 1997).

1.1.6.4. Ethylene

H2C=CH2 is the structure of ethylene. Unlike the other plant hormones, ethylene is a gaseous hormone, and the only member of its class. Of all the known plant growth substances, ethylene has the simplest structure. It is produced in all higher plants and is usually associated with fruit ripening (Crozier *et al.* 2000). Ethylene is produced from methionine in essentially all tissues, but its production varies with the type of tissue, the plant species, and also the stage of development (Crozier *et al.* 2000). Following are some of the responses known for ethylene (Crozier *et al.* 2000):

- Stimulates the release of dormancy.
- Stimulates shoot and root growth and differentiation (triple response).
- May have a role in adventitious root formation.
- Stimulates leaf and fruit abscission.
- Stimulates Bromeliad flower induction.
- Induction of femaleness in dioecious flowers.
- Stimulates flower opening.
- Stimulates flower and leaf senescence.
- Stimulates fruit ripening.

1.1.6.4.1. Ethylene Affects on Hairy Roots Secondary Metabolism

Literature reporting on the affects of exogenous ethylene on hairy root cultures is virtually non-existent. Perhaps this is due to the fact that ethylene alters the *in vitro* behavior of the plant tissues and promotes senescence (Kumar *et al.* 1996). Ethylene can promote or inhibit, in a species dependent fashion, the production of secondary products (Kumar *et al.*

1996). Shibli *et al*. (1999) reported that increasing ethylene content in the medium caused a reduction in pigment production and callus growth in *Vaccinium pahalae*. Fulzele *et al.* (1995) concluded that exogenous addition of ethylene to the culture medium stimulated terpenoid synthesis in *A. annua* L. plantlet cultures grown in a bioreactor. In our lab Bunk (1997) observed clone specific results in *A. annua* hairy roots supplied with ethylene (as ethephon). Whereas clone YUT-16 grew at or significantly above what was seen in control roots, *A. annua* clone YUM-290 grew unaffected in the presence of ethylene. In terms of secondary metabolites production, Bunk's data suggested that high concentrations of ethephon promoted high levels of artemisinin production (Bunk, 1997). When *A. annua* clone YUT-16 was provided with 5.0 or 15.0 mg/l ethylene (as ethephon), roots often grew poorly usually developing dense callused tissue or suspension cells.

1.1.6.5. Abscisic acid

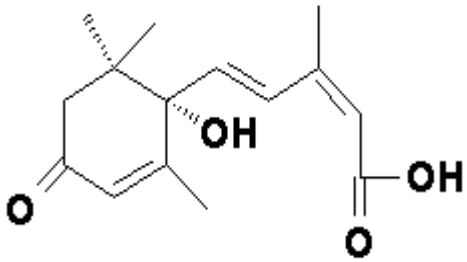

Figure 1.6.. Structure of abscisic acid, (taken from Srivastava,(2002).

Like ethylene, abscisic acid is a hormone that occurs as only one chemical structure (Figure 1.6). ABA is a naturally occurring compound in plants. It is a sesquiterpenoid (15-carbon) that is thought to be produced in the mevalonic acid pathway and from the cleavage of C_{40} precursors (Crozier *et al.* 2000). In whole plants biosynthesis primarily occurs in the leaves. The production of ABA is accentuated by stresses such as water loss and freezing temperatures. It is believed that biosynthesis occurs indirectly through the production of carotenoids. Following are some of the responses known for abscisic acid (Crozier *et al.* 2000).

- Stimulates the closure of stomata (water stress brings about an increase in ABA synthesis).
- Induces gene transcription especially for proteinase inhibitors in response to wounding which may explain an apparent role in pathogen defense.
- Inhibits shoot growth but will not have as much affect on roots or may even promote growth of roots.
- Has some effect on induction and maintenance of dormancy.
- Induces seeds to synthesize storage proteins.
- Inhibits the affect of gibberellins on stimulating *de novo* synthesis of α-amylase.

1.1.6.5.1. Abscisic acid Affects on Hairy Roots Secondary Metabolism

To my knowledge, little is known about the affects of exogenous ABA on hairy root culture. There are some reports, however, showing that ABA can inhibit secondary product accumulation but not hairy root morphology. In contrast, exogenous application of ABA to cell suspension cultures appears to stimulate secondary product accumulation, while inhibiting biomass yield in a time dependent manner. For example, the addition of 5 mg of abscisic acid per liter after 12 days growth of *T. chinensus* suspension cultures boosts paclitaxel accumulation roughly 5 times that of the controls (Luo *et al.* 2001). They also observed decreases in biomass in all cultures where ABA was added on day 0. Conversely, cultures where ABA was added at day 12 displayed minimal affects on biomass. On the other hand,Vanhala *et al.* (1998) demonstrated that exogenous application of ABA strongly inhibited hyocyamine accumulation in hairy root cultures of *H. muticus.* They did not observe any adverse effect on root biomass. Preliminary studies by Bunk (1997) in our lab showed that increasing concentrations of ABA in culture medium stimulated growth by as

much as 700% in *A. annua* hairy roots. Artemisinin levels were also elevated in the presence of 5.0 mg/l ABA (Bunk, 1997).

1.1.7. Combinatorial Phytohormone Treatment

The influences of these growth regulatory compound are numerous and far reaching. Studied individually, these hormones have increased our understanding of factors that affect growth and secondary metabolism of *in vitro* plant cultures. The information gained from the experiments above will inevitably guide the field of metabolic engineering in the future. However, one immediate objective, thus far overlooked by others, is the employ of hormone combinations in order to increase biomass and secondary metabolite production in hairy root cultures. Auxin:cytokinin combinations have met with success in a limited number of plant species. To my knowledge, however, no evidence of a multi-factorial study investigating the effects of phytohormones on secondary metabolism has been published. Since hormones regulate a multiplicity of plant functions, it is reasonable to assume that combination of hormones at different concentrations may also affect terpenoid biosynthesis.

In fact, a preliminary factorial study in our laboratory performed by Tara Smith (unpublished data, Table 1.1) showed the effectiveness of 27 phytohormone combinations on artemisinin and root growth in *Agrobacterium* transformed hairy root cultures of *A. annua* clone YUT-16. Each combination contained GA_3, ethephon, ABA and NAA component at 3 different concentrations, and was applied to hairy roots grown in modified B5 medium that

Table 1.1. Growth and artemisinin production in hairy roots of *Artemisia annua* in the presence of a 4-hormone combination in medium B-15 (Tara Smith, unpublished *data*).

Experiment number	*GA_3 (mg/L)*	*Ethephon (mg/l)*	*ABA (mg/l)*	*NAA (mg/l)*	*Fresh Wt (g)*	*Artemisinin (μg/g FW)*
1	0.005	5	0.1	5	2.27± 0.42	3.9 ± 0.1
2	0.005	5	5	0.01	0.38 ± 0.05	0
3	0.005	5	10	1	0.35 ± 0.08	0
4	0.005	5	0.1	1	0.85 ± 0.15	0
5	0.005	10	5	5	0.29 ± 0.01	0
6	0.005	10	10	0.01	0.13 ± 0.01	0
7	0.005	15	0.1	0.01	0.2 ± 0.03	0
8	0.005	15	5	1	0.13 ± 0.02	0
9	0.005	15	10	5	0.14 ± 0.02	0
10	0.01	5	0.1	1	1.09 ± 0.18	3.9 ± 0.1
11	0.01	5	5	5	0.43 ± 0.1	0
12	0.01	5	10	0.01	0.25 ± 0.04	0
13	0.01	10	0.1	0.01	0.41 ± 0.04	0
14	0.01	10	5	1	0.33 ± 0.01	0
15	0.01	10	10	.5	0.25 ± 0.01	0
16	**0.01**	**15**	**0.1**	**5**	**0.46 ± 0.03**	**9.5 ± 1.9**
17	0.01	15	5	0.01	0.15 ± 0.02	0
18	0.01	15	10	1	0.13 ± 0.01	0
19	0.02	5	0.1	0.01	1.31 ± 0.18	4.2 ± 0.5
20	**0.02**	**5**	**5**	**1**	**0.49 ± 0.17**	**9.45 ± 2.1**
21	**0.02**	**5**	**10**	**5**	**0.47 ± 0.15**	**10.2 ± 2.1**
22	0.02	10	0.1	0.5	1.27 ± 0.07	4.2 ± 0.6
23	0.02	10	5	0.01	0.18 ± 0.03	0
24	0.02	10	10	1	0.21 ± 0.02	0
25	0.02	15	0.1	1	0.42 ± 0.06	5.9 ± 5.1
26	0.02	15	5	5	0.25 ± 0.05	0
27	0.02	15	10	0.01	0.11 ± 0.02	0

Note**: All experiments run in autoclaved medium B15 (Appendix A1).**

were previously optimized for growth (Weathers *et al*., 1996; Weathers *et al.,* 1997). That optimized medium is defined as B15. Interestingly, three of the twenty-seven experiments run yielded the highest levels of artemisinin in her study. Unfortunately, this increase in artemisinin was also accompanied by low root biomass accumulation (Table 1.1). These data suggest that use of phytohormone combinations may be a potential means of increasing secondary product accumulation. It should be noted that these preliminary experiments were run without hormone-free controls, and in autoclaved medium. Hence, it is not known whether this system stimulated production of artemisinin above B15 or B5 controls. The process of autoclaving also variably hydrolyzes sugars, and removes essential ammonia from the medium (De Jesus and Weathers, unpublished).

1.1.8. Multi-stage Culture Process

It was observed some time ago that many secondary metabolites are often produced after most growth has ceased, or at least not during exponential phase (Bourgaud *et al*. 2001). Indeed the first commercially produced plant product, shikonin, is produced during a second stage, in a new medium, and after growth has mainly ceased (Yazaki *et al*., 1999). This process is known as a two-stage culture and in some cases, growth and secondary metabolite production has been as high as five times that of a one-staged culture that used only an original medium formulation (Toivonen *et al.* 1991; Jung *et al.* 1994; Luo *et al.* 2001).

In our laboratory, orthogonal multi-factorial design experiments incorporating inorganic salts and sugar, along with inoculum age were performed to obtain an optimum growth medium for *A. annua* hairy roots (Weathers *et al.* 1997). Unfortunately, the same conditions were not optimal for artemisinin production (Weathers *et al.* 1997). We now

know that time of addition of nutrients, hormones, or elicitors, also plays a key role in secondary metabolism. For example, Luo *et al.* (2001) observed in *T. chinensus* a dramatic decrease in root biomass occurred when phytohormones were added at day 0 as opposed to addition at day 12. Used together a two-staged addition of plant hormones should begin to allow for the optimization of high growth and secondary metabolite production conditions.

1.1.9 Summary

Plant derived therapeutics represent one quarter of a multi-billion dollar global pharmaceutical industry (GEN, 1998). Terpenoids, which are the largest group of these products, have been extensively used but with little understanding of terpenoid biosynthesis (van der Hoevena *et al*. 2000; Chappell, 1995). A considerable amount of work remains to be done in order to determine what controls terpenoid biosynthesis.

Recent novel genetic work involving the introduction of key regulatory enzymes in the two pathways, as well as cDNA library screening studies has greatly improved a fundamental understanding of terpenoid biosynthesis (Croteau *et al.* 2000; Eisenreich *et al*, 1998; Souret, 2002). However, these techniques are costly and time-consuming and often result in only slight improvement, if any, of secondary metabolite yields. *In vivo* optimization strategies using field-grown crops harvested annually, invariably return poor yields with respect to amount produced of a plant-derived drug per plant hectare to the amount needed by an afflicted person (Dhingra *et al.* 2000). Researchers using *in vitro* cultures, dealing with transformed hairy roots and suspension cells, have recorded significant increases in the production of secondary compounds as well as in growth of whole culture or specific aspects of the cultures (Dhingra *et al.* 2000). In our laboratory, the use of medium

optimization, light, and phytohormones, such as GA, ABA, IAA, NAA, ethylene, and cytokinin have also shown some promising results.

Of the techniques mentioned above, the use of hairy root cultures, medium optimization, and the addition of phytohormones are germane to the following research. Our laboratory uses the hairy root clone YUT-16 that was selected for its fast growth rate and artemisinin production levels (Weathers *et al.* 1994). The following research report is based on the use of an optimized growth medium (B15; Weathers *et al.* 1997), coupled with a four factor fractional factorial design set of experiments (T. Smith, unpublished, Table 1), to investigate optimization of growth and artemisinin production in transformed roots of *A. annua.*

CHAPTER 2

RESEARCH OBJECTIVES

It was expected that an effective culture process can be designed for *Agrobacterium* transformed hairy roots of *A. annua*, whereby artemisinin production can be optimized, using a two-stage process approach and phytohormone addition. Hence, the goal of my research was to investigate and compare the effects of exogenously applied phytohormones on growth and secondary metabolite production in hairy roots of *A. annua* using a two-stage culture system. Specifically my aims were to:

- Measure the individual effects of exogenously applied auxins, cytokinins, abscisic acid, gibberellic acid, and ethephon on single root tip and bulk *A. annua* root growth.
- Measure the individual effects of exogenously applied auxins, cytokinins, abscisic acid gibberellic acid, and ethephon on artemisinin production in bulk *A. annua* roots.
- Measure the combinatorial effects of exogenously applied phytohormone combinations 16, 20 and 21 (Table 1) on growth and artemisinin production in bulk *A. annua* roots.
- Analyze time of addition effects of exogenously applied phytohormone combinations 16, 20 and 21 (Table 1) on growth and artemisinin production in bulk *A. annua* roots.
- Investigate concentration of hormone-to-root inoculum effects on growth and artemisinin production in bulk *A. annua* roots provided with a combination of phytohormones.
- Investigate fresh medium and physical manipulation effects on growth and artemisinin production in bulk *A. annua* roots provided with a combination of phytohormones.
- Determine peak growth and artemisinin production in bulk *A. annua* roots provided with a combination of phytohormones.

CHAPTER 3

3.0. METHODS

3.1.1. Hairy Root Initiation and Culture Conditions

The hairy root clone YUT16 of *Artemisia annua L.* (Weathers *et al.* 1994) was used in all experiments. A half gram of roots was subcultured every 14 days and cultures were maintained in 50 ml of autoclaved Gamborg's B5 basal medium (Gamborg *et al.* 1968) supplemented with 3% (w/v) sucrose at pH 5.7 in 125 ml Erlenmeyer shaker flasks under continuous cool white fluorescent light (2-5 μmol M^{-2} s^{-1}) at 25 º C on an orbital shaker at 100 rpm (Labline Instruments Inc., Melrose Park, IL). For experiments, 0.5 g fresh weight of healthy root tissue (transparent white) from 14-day-old cultures, grown as above, was inoculated into 50 ml of filter sterilized (0.22μm Vacucap™ 60 filter, Pall-Gelman Sciences Ann Arbor, MI) Gamborg's B5 basal medium at pH 5.7 and with 3% (w/v) sucrose.

3.1.2. Individual Effects of Phytohormones on Hairy Root Cultures: In Flasks

To gauge the affects of individual hormones on biomass accumulation and artemisinin production, the following filter sterilized hormones (0.22 μm Acrodisc™ syringe filters, Pall-Gelman Sciences Ann Arbor, MI) were added individually to roots grown in flasks: ABA, 5-1-hydroxy-2-6-6-trimethyl-4-oxocyclohex-2-en-lyl-3-methyl-[2E,4E]-pentadienoic acid; ethephon, 2-chlorophosphonic acid; GA_3, acetyl gibberellic acid; NAA, α-napthalene acetic acid ; IAA, 3-indole acetic acid; and BAP, benzylaminopurine (all from Sigma Aldrich Chemical, St. Louis, MO). The experiments were run using medium B5 as described above with an inoculum of 0.5 grams fresh weight of healthy root tissue in each

flask. Cultures were grown in triplicate. Hormone concentrations were: ABA at 0.1, 5.0 and 10.0 mg/l; ethephon at 5.0, 10.0 and 15.0 mg/l; GA_3 at 0.005, 0.01 and 0.02 mg/l; NAA at 0.1, 0.5 and 1.0 mg/l; IAA at 0.1, 0.5 and 1.0 mg/l; BAP at 0.5 and 1.0 mg/l. Hormones were added at the day of inoculation. All cultures were harvested after 14 days of growth and the artemisinin and biomass accumulation were measured as described below.

The effects of adding single hormones of ABA at the day of inoculation and followed by NAA at day 14 in an effort to determine whether or not this combination of hormones stimulated high levels of artemisinin as well as growth. The experiments were run in medium B5 as above with a fresh weight inoculum of 0.5g. Roots were supplemented with 1.0 mg/l ABA at inoculation and grown for 14 days. After 14 days of growth, the medium was drained and fresh medium was added to the cultures along with 0.1 mg/l of NAA, and roots were allowed to grow for an additional 3 days. Cultures that were grown in medium B5 with an equal amount of inoculum were used as controls.

3.1.3. Individual Effects on Single Roots: In Six-Well Plates

To monitor the affects of individual hormones on growth, single hormones were added to 2.5cm root tips cultured in polystyrene six-well plates (Multiwell™ Primaria™ 6 well, Becton Dickinson® Bedford, MA) according to Srinivasan *et al.* (1997). It is also possible to reuse the 6-well plates by resterilizing them (See Appendix A2). Controls contained only B5 medium. A single ~ 2.5 cm root tip was inoculated into each of the six wells containing 5 ml of B5 medium supplemented with a single, filter-sterilized hormone at the day of inoculation as follows: ABA at 0.1, 5.0 and 10.0 mg/l; ethephon at 5.0, 10.0 and 15.0 mg/l; GA_3 at 0.005, 0.01 and 0.02 mg/l; NAA at 0.1, 0.5 and 1.0 mg/l; IAA at 0.1, 0.5

and 1.0 mg/l; and BAP at 0.5 and 1.0 mg/l. These hormones and respective concentrations were chosen based on earlier experiments performed by Bunk (1996) and (T. Smith, unpublished data, Table 1.1). Roots were harvested 14 days later and the number of lateral roots, length of each lateral, the sum of all lateral lengths, and length of the primary root, the lateral root density per centimeter, and the root growth unit (RGU). The root growth unit is a measurement of lateral root formation on growing roots. The RGU is defined as the sum of the length of all lateral roots and the length of the primary root divided by the total number of root tips; the higher the RGU the higher the number of lateral roots being produced, and vice versa (Srinivasan *et al.* 1997, Wyslouzil *et al*. 2000; Yu and Doran, 1994).

(RGU) = (∑ length of the laterals + length of primary root) (Number of root tips)$^{-1}$

Data were statistically analyzed using ANOVA™ software. Each plate contained six replicates per experiment for each hormone treatment, and each experiment was repeated.

3.1.4. Hormone Combination Effects on Single Roots: In Six-Well Plates

To determine how a mixture of hormones affects root growth, single roots were inoculated into six-well plates containing hormone combinations 16, 20, and 21 shown in Table 1.1. The previous fractional factorial experiments described in (Table 1.1) showed that combinations Nos. 16, 20 and 21, prepared in B15 medium, yielded the highest levels of artemisinin in her study, but resulted in poor growth. The combinations above were also used to grow roots in six-well plates, but in B15 medium with hormones added at the day of inoculation. Medium B15 contains 5% (w/v) sucrose and 1.0 mM and 15 mM of the phosphate and nitrate salts, respectively, found in normal B5 medium (Appendix A1). In a subsequent experiment, phytohormones were added to single roots, as above, but after 14

days of growth, and cultures were then harvested 3 days later at day 17. Controls containing either B5 or B15 medium, with no hormones, were also harvested at day 14 and at day 17. Growth was measured as described for the single hormone six-well plate experiments.

3.1.5. Two-Stage Cultures

A two-stage culture system was developed to study the affects of medium composition and timing of hormone treatment on root growth and artemisinin production (Figure 3.1). Experiments were run in flasks using both medium B5 and B15 as controls, and B15 containing three different filter sterilized hormone combinations hereafter referred to as H16, H20 and H21 (Table 1.1). Hormones were added either at the day of inoculation or at day 14. In previous fractional factorial experiments, combinations Nos. 16, 20, and 21 (Table 1.1) yielded the highest levels of artemisinin. When hormones were added at inoculation, harvest was at day 14 (Figure 3.1). When hormones were added at day 14, harvest was at day 17 (Figure 3.1). In a third experiment, roots were provided hormones at day 14, but in fresh medium, and incubated for three days post-phytohormone addition and harvested on day 17. This experiment was done to build on the results of the prior two-stage experiments and on earlier work of Weathers *et al.* (1997) that showed maximum artemisinin production occurred early after subculture. Each combination in each experiment was run in triplicate and each experiment was repeated. Controls containing either B5 or B15 medium, without hormones, were also harvested at day 14 and at day 17.

Combination/Control	Hormone Addition and Harvest (day)
B5 (Control (No hormones)	0----------14 harvest 0----------14----------17 harvest
B15 (Control (No hormones)	0----------14 harvest 0----------14----------17 harvest
B15 + Hormone H 16	0----------14 add hormones harvest 0----------14----------17 add hormones harvest
B15 + Hormone H 20	0----------14 add hormones harvest 0----------14----------17 add hormones harvest
B 15 + Hormone H 21	0----------14 add hormones harvest 0----------14----------17 add hormones harvest

Figure 3.1. Experimental design combinations under which the two-stage experiment was run. Three replicates were run for each combination. Hormone combinations added to the cultures represent those combinations that produced the highest artemisinin levels found in Table1.

3.1.6. Hormone-to-Biomass Ratio Experiments

3.1.6.1. Inoculum Size Effects on artemisinin and growth

To determine whether or not the ratio of hormone-to-root mass was critical to growth and artemisinin production, a series of experiments was run with one of three hormone concentrations: the original, ten times the original or 0.1 times the original phytohormone concentration of H16, H20 and H21 (Table 1.1). Cultures fed hormones at day zero were inoculated with 0.5 grams of root tissue along with either the concentration of hormone H16, H20 or H21 at the level shown in (Table 1.1) or at one-tenth that level. These two hormone-to-biomass ratios are termed 10 and 1x, respectively. Cultures fed hormones after 14 days of growth were assumed to have 5.0 grams of tissue and were fed hormone H16, H20 or H21 at concentrations equal to or ten times the level shown in (Table 1.1). These two hormone-to-biomass ratios are termed 1x and 10x, respectively (Figure 3.2).

3.1.6.2. Fresh Medium Effects on Artemisinin and Growth

In a subsequent experiment, I further examined the effects of combination No. 16 on root tissue that had been inoculated into fresh medium at day 14 in an effort to confirm whether or not this combination, indeed, stimulates highest levels of artemisinin. The experiments were run in medium B15 as above with a fresh weight inoculum of 5.0 g. Roots were not provided with hormones at inoculation and grown in medium B15 for 14 days. After 14 days of growth, the medium was drained and fresh medium was added to the cultures along with H16 and then the roots were allowed to grow for an additional 3 days prior to harvest at day 17. Cultures were also grown in medium B15 under the same conditions without being provided H16 at day 14.

Combination/Control	**Hormone Addition and harvest (day)**
B5 (Control (No hormones)	0----------14 harvest
	0----------14----------17 harvest
B15 (Control (No hormones)	0----------14 harvest
	0----------14----------17 harvest
B15 + Hormone H16	0----------14 add hormones (0.1x or 1x) + harvest 5.0 gram of root tissue
	0----------14----------17 (1x or 10x) add hormones + harvest 5.0 gram of root tissue
B15 + Hormone H20	0----------14 add hormones (0.1x or 1x) + harvest 5.0 gram of root tissue
	0----------14----------17 (1x or 10x) add hormones + harvest 5.0 gram of root tissue
B 15 + Hormone H21	0----------14 add hormones (0.1x or 1x) + harvest 5.0 gram of root tissue
	0----------14----------17 (1x or 10x) add hormones + harvest 5.0 gram of root tissue

Figure 3.2. The combinations under which the hormone concentration to biomass ratio experiments were run. Three replicates were run for each combination. Hormones added at day 0 are added at concentrations of either one tenth or at the original concentration run in the two-stage culture experiment (Figure 3.1, Table 1.1). Those added at day 14 are in concentrations of either the original or ten times those in (Table 1.1).

3.1.7. Fresh Medium and Manipulation effects on Artemisinin and Growth

In order to determine if there is both a fresh medium and physical manipulation effect on artemisinin production and biomass accumulation, roots were supplemented with no hormones at inoculation and grown in medium B15 for 14 days in 2.8-liter Fernbach flasks. After 14 days of growth, the medium was drained and saved. The roots were rinsed 3x with distilled sterile water to wash residual medium from the roots. Five-gram aliquots of fourteen-day-old roots, from the Fernbach flasks, were chopped or gently transferred, blotted, weighed and inoculated into 125 ml culture flasks on day 14. Either fresh medium or the old medium, saved from the prior growth, was added to 125 ml culture flasks cultures along with the original phytohormone concentration found in H16 (Table 1.1). These cultures were allowed to grow for an additional three days prior to harvest at day 17. Control cultures were grown in medium B15 under the same conditions but without being provided H16 at day 14.

3.1.8 Determination of Peak Artemisinin Production Time

In order to determine when the maximum artemisinin production occurred post-hormone addition, roots were harvested every two days, post-hormone addition, for six days. Roots were supplemented with no hormones at inoculation and grown in medium B15 for 14 days in 2.8-liter Fernbach flasks. After 14 days of growth, the medium was drained and saved. The roots were rinsed 3x with distilled sterile water to wash residual medium from the roots. Five grams of fourteen-day-old roots, from the Fernbach flasks, were chopped, blotted, weighed, and inoculated into 125 ml culture flasks on day 14. Fresh medium was added to 125 ml culture flasks along with the original concentration of phytohormones found in H16 (Table 1). The roots were allowed to grow for an additional six days. Three random

cultures were harvested every two days post-hormone addition until day six. Control cultures were grown in medium B15 under the same conditions without being provided H16 at day 14.

3.1.9 Biomass Determination and Artemisinin Extraction

Harvested roots were rinsed three times with 15 ml of distilled water to remove medium salts, blotted gently to remove excess water, and finally weighed on a Mettler™ AE 163 electronic balance (Caley and Whitmore Corporation, Somerville, MA) to obtain their fresh weight. Artemisinin was extracted from two grams of roots with six ml of toluene (3 ml $gram^{-1}$) in 13 x 100 mm disposable borosilicate glass tubes (Fisherbrand® Pittsburg, PA), by sonication in a waterbath sonicator (FS60 Fisher Scientific,) for 30 minutes at 4 º C. After sonication the samples were centrifuged for ten minutes at 6 x g (HN-S centrifuge, International Equipment, Needham Heights, MA) and the supernatant was decanted. The extraction was repeated twice again with fresh toluene, and the supernatants were pooled and dried under nitrogen. The dried samples can be stored indefinitely at –20 º C in covered borosilicate glass tubes until high pressure liquid chromatography (HPLC) analysis.

3.2.0 HPLC Analysis of Artemisinin

Artemisinin was assayed by HPLC (Waters Associates, Milford, MA) as described by Smith *et al.* (1997). The samples were hydrolyzed to the Q260 derivative as follows. Dried toluene extracts were resuspended in 100 µl of methanol and 400 µl of 0.2% NaOH (w/v), mixed thoroughly and heated for 35 minutes at 50 º C. The reaction was quenched with 100 µl of methanol and 400 µl of 2.0 % acetic acid (w/v) to bring the sample to 1.0 ml. The

samples were mixed thoroughly and immediately placed on ice to stabilize them. The samples were filtered (0.22 µm FP-200 13mm FP-Vericel™ membrane filter, Pall-Gelman Laboratory) and run on a reverse phase C-18 HPLC column (15 cm Microsorb-MV™ C-18 column with a 4.6 mm i.d. and containing 5 um beads of 100 Å pore size, Varian Analytical Instruments, Walnut Creek, CA) at 1 ml min^{-1}. The mobile phase contained 0.22µm filtered 0.01 M phosphate buffer pH 7.0 and methanol (55:45, v/v). After mixing, the pH of the mobile phase was again adjusted to 7.0 with 0.4 N HCL. Artemisinin was quantified based on an external standard (Sigma-Aldrich, St. Louis, MO).

3.2.1. Verification of Artemisinin Concentrations

Co-injection was used in order to positively identify peaks as artemisinin. Suspected artemisinin peaks were noted, and their area recorded from the original sample that was run. An aliquot of a concentrated, Q260 derivatized, filtered (0.22 µm FP-200 13mm FP-Vericel™ membrane filter, Pall-Gelman Laboratory) artemisinin standard (Sigma-Aldrich, St. Louis, MO) solution was added to the analyzed sample to achieve an artemisinin peak area about four times that of the area of the putative artemisinin peak. If the putative peak increased in area to an amount that was 4-5 times its original area, the peak was determined to be artemisinin. Quantitation was based on the original injection prior to the addition of the standard.

CHAPTER 4

4.0. RESULTS

4.1. Individual Phytohormone Effects on Root Growth

In the previous experiments shown in Table 1.1, three combinations of phytohormones yielded increased levels of artemisinin while severely inhibiting the growth of the root cultures. Moreover, to my knowledge, the individual affects of NAA, GA_3, ethephon, ABA, and BAP on hairy root development of *A. annua* have not been previously reported. Thus, it was useful to investigate the individual roles that these phytohormones have on root growth. Growth was measured of both bulk roots, in flasks, and single roots grown in six-well plates.

4.1.1. Single Root Responses

Six-well polystyrene plates were used to monitor the affects of the individual phytohormones on the growth of single roots after 14 days of growth. Within each hormone concentration series, the yield of root mass usually decreased as the concentration increased (Table 4.1). Single roots grown in medium B5 with 0.01 mg/l GA_3 produced the highest values in terms of the number of lateral roots, length of the primary root, lateral root tip density, total lateral root length, and total root length. However, roots grown in GA_3 did not have the highest RGU. Roots grown in IAA at concentrations of 0.01 and 1.0 mg/l had essentially the same RGU values as those grown in either 0.01 mg/l GA_3 or 0.02 mg/l GA_3, and all concentrations of ABA. High RGU values indicate a high level of lateral branching and low RGU values represent low levels of lateral branching. Roots in each hormone

Table 4.1 Growth response of single root tips grown for 14 days in medium B5 with hormones provided at different concentrations.

Root Characteristic	*B5*	*Hormone concentration (mg/l)*							
		NAA 0.01	*NAA 1.0*	*NAA 5.0*	*IAA 0.01*	*IAA 1.0*	*IAA 5.0*	*GA 0.01*	*GA 0.02*
No. of laterals	8.00	9.50 a,m	8.7 b,m	4.67 c	30.71 a	25.50 b	13.50 c	94.67 a,x	62.17 b
Length of Primary (cm)	3.20	2.86 a	2.6 b	2.50 c	2.80 a	2.83 a	2.53 b	7.13 a,x	5.37 b
Lateral Density (lat/cm)	2.20	3.23 a,m	3.40 a	1.87c	10.69 a	8.57 b	5.31 c	13.51 a,x	11.68 b
Total Length of Laterals (cm)	7.87	7.00 a,m	4.63 b	1.20 c	21.80 a	17.65 b	2.43 c	84.77 a,x	54.17 b
AVG Lateral Length (cm)	1.33 x	0.67 a	0.46 b	0.22 c	0.78 a	0.72 a	0.16 c	0.97 a	0.56 b
Total Root Length (cm)	11.07	9.87 a	7.18 b	3.70 c	24.60 a	20.48 a	4.97 c	91.90 a,x	59.53 b
RGU	0.77	0.75 a,m	0.65 b,m	0.32c	0.90 a,x	0.88 a,x	0.50 c	0.97 a,x	0.96 b,x

Root Characteristic	*B5*	*Hormone concentration (mg/l)*							
		ABA 1.0	*ABA 5.0*	*ABA 10.0*	*ETHE 5.0*	*ETHE10.0*	*ETHE 15.0*	*BAP 0.5*	*BAP 1.0*
No. of Laterals	8.00	56.00 a	31.67 b	16.50 c	5.67 a	5.83 a	5.50 a	12.20a	7.13 b
Length of Primary (cm)	3.20	5.03 a	3.10 b,m	2.22 c	2.50 a	2.50 a	2.50 a	2.60 a	2.54 b
Lateral Density (lat/cm)	2.20	10.84 a	8.37 b	5.08 c	2.27 a,m	2.33 a,m	2.20 a,m	4.51a	2.67 b
Total Length of Laterals (cm)	7.87	54.43 a	31.45 b	13.67 c	1.85 a	1.33 b	1.33 b	1.83 a	0.86 b
AVG Lateral Length (cm)	1.33 x	1.00 a,m	0.73 b	0.55 c	0.32 a	0.23 b	0.25 b	0.15 a	0.12 b
Total Root Length (cm)	11.07	59.47 a	34.55 b	15.88 c	4.35 a	3.83 b	3.83 b	3.45 a	3.91 b
RGU	0.77	0.96 a.x	0.93 a,x	0.84 b,x	0.43 a	0.35 b	0.35 b	0.48 a	0.40 b

Note: The letters "a, b, and c" represent significant differences between roots within a hormone concentration series; the letter "m" indicates statistical similarity when comparing roots grown in each hormone concentration to the medium B5 controls; "x" represents the maximum value when comparing all hormone concentrations and the B5 control roots. Statistical test was done using ANOVA™ at $p<0.05$.

concentration series, other than ethephon and BAP, produced higher growth measurements than the B5 controls, except for average lateral root length (Table 4.1).

4.1.2. Bulk Growth

The hormone IAA was tested in concentrations equivalent to those for NAA. This was done to determine whether or not the natural auxin, IAA, was more effective in affecting growth than the synthetic NAA. Roots provided with either NAA or IAA appeared to yield comparable levels of root biomass (Figure 4.1). Generally, biomass was inversely proportional to the concentration of phytohornmone for each of the six hormones tested (Figure 4.1). Those cultures incubated in ethephon yielded the lowest root growth of all control and experimental flasks assayed and appeared very callused and dark brown after 14 days. All cultures provided with ABA produced the highest biomass levels. One and 5 mg/l concentrations of ABA stimulated the highest production of root biomass (Figure 4.1). In contrast, roots cultured in BAP grew poorly.

4.1.3. Phytohormone Combination and Time of Addition Affects Growth

Earlier work by Bunk (1997) had suggested that the hormones GA3, ethephon, ABA, and NAA would stimulate growth of *A. annua* hairy roots. Subsequent multi-factorial experiments by Smith (Table 1.1) showed that hormone addition at the time of inoculation adversely affected root growth. Consequently, it seemed reasonable to determine what effect later addition of phytohormones had on root growth and artemisinin production.

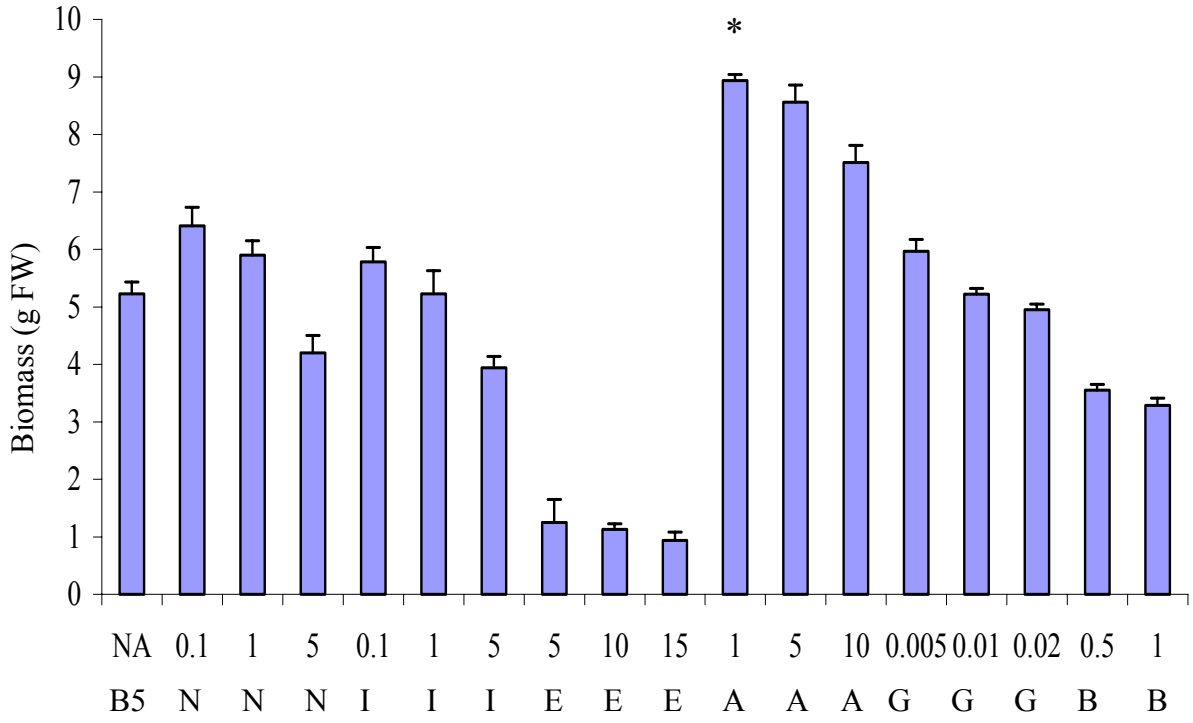

Figure 4.1. The effects of individual phytohormones added at day 0 on growth in cultures harvested after 14 days of growth.. N, NAA; I, IAA; E, ethephon; A, ABA; G, gibberellic acid; B, BAP; B5, B5 control; NA, no addition. The "*", represents the highest overall value. Concentrations on the x-axis represent mg/l quantities of hormones added.

4.1.3.1. Single Root Responses to Time of Hormone Addition

In order to determine whether or not time of addition plays an important role in biomass accumulation, a modified version of the previous experimental six-well experiment was run. Growth was measured after single roots were incubated in hormone H16, H20 and H21 (Table 1.1) provided either at the day of inoculation or at day 14 (Table 4.2).

Table 4.2. Growth response of single root tips grown for either 14 or 17 days in medium B15 including H 16, H20 and H21 found in Table 1.1.

Root Characteristic	Hormone addition at Day 0			
	B15 N/H	***H16***	***H21***	***H20***
No. of Laterals	12.67 x	5.17 a	7.00 b	8.33 c
Length of Primary (cm)	4.03 x	2.50 a	2.50 a	2.50 a
Lateral Density (lat/cm)	3.25 x	2.07 a	2.80 b	3.33 c,x
Total Length of Laterals (cm)	10.12 x	0.70 a	0.70 a	0.83 a
AVG Lateral Length (cm)	0.75 x	0.14 a	0.10 a	0.10 a
Total Root Length (cm)	12.53 x	3.20 a	3.20 a	3.33 a
RGU	0.35 x	0.22 a	0.22 a	0.25 a

Root Characteristic	Hormone addition at Day 14			
	B15 N/H	***H16***	***H20***	***H21***
No. of Laterals	12.87	15.23 a,x	12.35 b	12.51 b
Length of Primary (cm)	4.00 x	4.10 m,a,x	3.95 m,a,x	3.95 m,a,x
Lateral Density (lat/cm)	3.31	3.68 a,x	3.09 b	3.02 b
Total Length of Laterals (cm)	10.24	14.95 a,x	12.52 b	13.80 c
AVG Lateral Length (cm)	0.81	1.00 a,x	0.95 a,x	0.85 m, b
Total Root Length (cm)	12.75	14.23 a,x	9.62 b	9.25 b
RGU	0.40 x	0.45 m,a,x	0.36 m,b,x	0.38 m, b,x

Note: The letters "a, b, and c" represent statistically significant differences between H16, H20 and H21; "m" indicates statistical similarity when comparing the roots grown in each hormone concentration to the medium B15 controls; "x" refers to the maximum value of all the hormone combinations and the B15 control. N/H = No hormones provided. Statistical test done using ANOVA™ at $p<0.05$**.**

Root tips provided with phytohormones at the day of inoculation (day 0) only produced higher numbers of laterals than those simply grown in medium B15. Indeed roots provided with hormone combinations at the day of inoculation were growth inhibited compared to the B15 control, except in terms of lateral density (Table 4.2). Roots supplemented with H16, H20 and H21 fourteen days post-inoculation, showed significantly more growth than the B15 controls at 17 days compared to cultures supplemented at the day of inoculation. These roots produced more laterals, longer laterals and primary roots, and a

greater overall root length than the medium B5 controls. Roots grown in H16 produced the highest root measurements overall (Table 4.2).

When root growth in B5 medium was compared to that in B15 medium, roots grown in medium B5 produced the highest growth response values in all root characteristics except length of the primary root, average lateral length, and the RGU (Table 4.3). While not true for all root characteristics of growth, roots cultured in medium B5 appeared to produce more root growth than those cultured in B15 after 14 days of growth (Table 4.3).

Table 4.3. Growth response of single root tips after 14 days in medium B5 or medium B15.

Root Characteristic	B5	B15
No. of Laterals	8.00 m	2.67
Length of Primary (cm)	3.20	4.03 m
Lateral Density (lat/cm)	2.20 m	0.92
Total Length of Laterals (cm)	7.87 m	3.03
AVG Lateral Length (cm)	1.33 m	1.24 m
Total Root Length (cm)	11.07 m	7.07
RGU	0.77 m	0.65 m

Note**:** The letter "m" indicates statistical difference when comparing the roots grown in medium B15 to roots grown in medium B5 after ANOVA™ at $p<0.05$.

4.1.4. Bulk Growth: Two-Stage Culture System

To examine whether or not time of hormone addition played an important role in biomass accumulation, a modified version of the previous experimental design (Figure 3.1) was run using the three hormone combinations, H16, H20, and H21 from table 1.1. Hormones were added to cultures either at day 0 or at day 14 and harvested at day 14 and 17, respectively. Cultures supplemented with phytohormones at the day of inoculation grew very poorly (Figure 4.2A). There was also rapid disorganization of the root matrix resulting in

callused tissue and suspension cells (data not shown). Although, H20 and H21 stimulated growth a little above that of roots cultured with H16, none of the cultures provided hormones at day 0 grew better than the B15 controls (Figure 4.2A). This was in agreement with earlier work performed in our laboratory (T. Smith, Table 1.1).

However, when cultures were provided hormones at day 14 of the culture cycle and allowed to grow an additional three days, those cultured in H16 stimulated the highest levels of root biomass compared to roots provided with either H20 or H21 and the 17 day B15 controls. This suggests that combination 16 is stimulatory to growth, but only when added later in the developmental cycle of the roots.

Cultures grown in medium B15 and harvested at day 14 grew better than the B5 controls. Both medium B5 and B15 control roots produced higher biomass levels than roots grown in H16, H20 and H21 when hormones were added at inoculation.

4.1.5. Effect of the Hormone-to-Biomass Ratio

At inoculation, the ratio of hormones to biomass provided in the previous experiments was about 10 times that produced upon hormone introduction at day 14. The poor growth observed in cultures fed hormones at day zero might, thus, be in response to the higher specific hormone concentration. Experiments were, therefore, conducted to test the effect of the hormone-to-biomass ratio on root growth.

To study whether or not the ratio of hormone-to-root mass was critical to growth, roots that were provided hormones at day zero and harvested at day 14. Hormones were supplied at the concentrations of H16, H20, or H21 at the level shown in Table 1.1, or

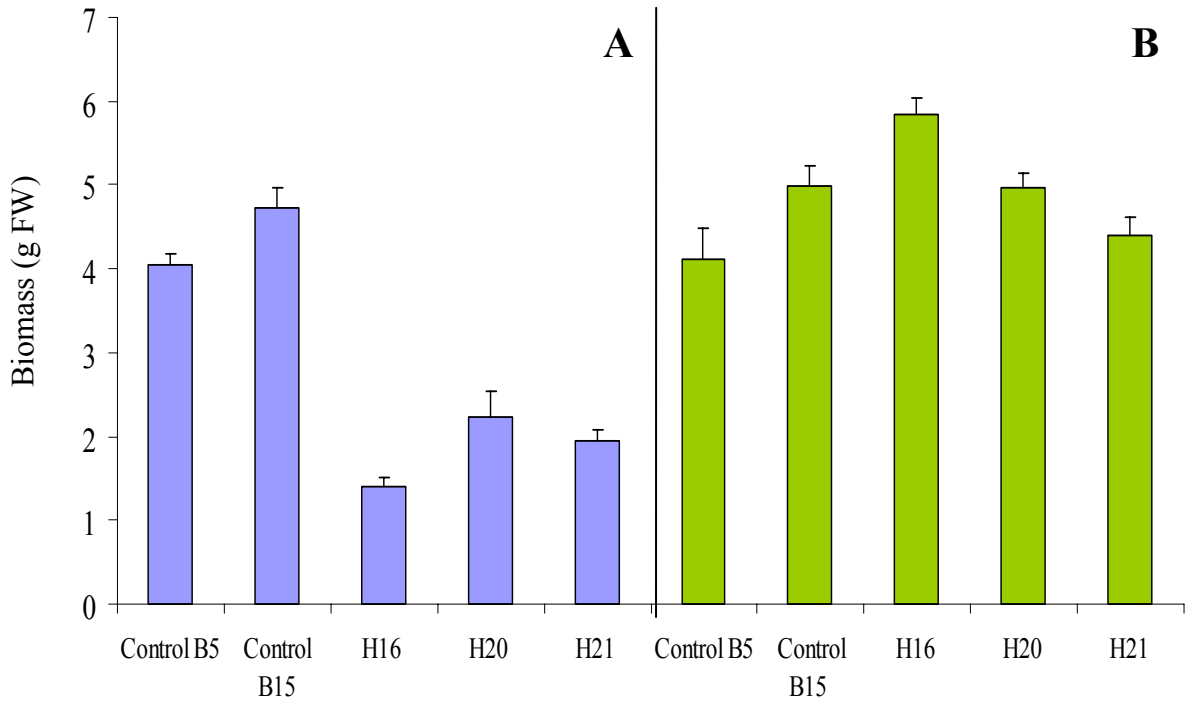

Figure 4.2 The effects of phytohormones on the biomass accumulation of hairy root cultures of *A. annua.* **A**, all cultures harvested after 14 days of growth; hormone cocktails No. 16, 20, and 21 were added at day zero to roots growing in B15 medium. **B,** all cultures were harvested after 17 days of growth; hormone cocktails No. 16, 20, and 21 were added at day 14 to roots growing in B15 medium and then were allowed to grow three days prior to harvest.

at one-tenth that level. Cultures provided hormones at day 14 and harvested at day 17 were provided hormones either at the level shown in Table 1.1 or at 10 times that level (Figure 3.2). In this way each group of roots was provided hormones at a ratio of 1:1 or 10:1 of biomass.

Cultures provided at day 0 with one-tenth the original hormone concentrations exhibited significant growth increases when compared to roots in which the original concentrations were used (Figure 4.3). Roots cultured in one-tenth the concentration of H16, H20 and H21 displayed roughly twice the biomass of their 1x counterparts. Compared to their B15 controls, however, root growth was still significantly less (Figure 4.3)**.** When 14-day-old root cultures were supplied with ten times the original phytohormone concentrations used in experiments shown in Table 1.1, growth was also inhibited compared to roots that were fed the original concentrations (Figure 4.4). Growth inhibition was particularly high for roots grown in H16 10x. Interestingly, roots grown in combination 21 showed no growth inhibition after the higher 10x concentration. Of particular interest is the result showing that roots incubated with original concentration, 1 x of combination 16, stimulated root growth 12 percent above the medium B15 controls. Taken together these results show that there is, indeed, a significant ratio effect of hormones on growth. However, other factors are also at play because hormones fed at day 14 stimulated root growth beyond the B15 controls. Decreasing the hormone concentration at inoculation was still inhibitory towards growth (Figure 4.3).

The roots that were provided with hormones on day 14 were not subjected to the stress of subculture. In fact, these cultures were 14 days removed from that stress. Therefore, it was conceivable that physical manipulation or addition of fresh medium would also likely influence growth.

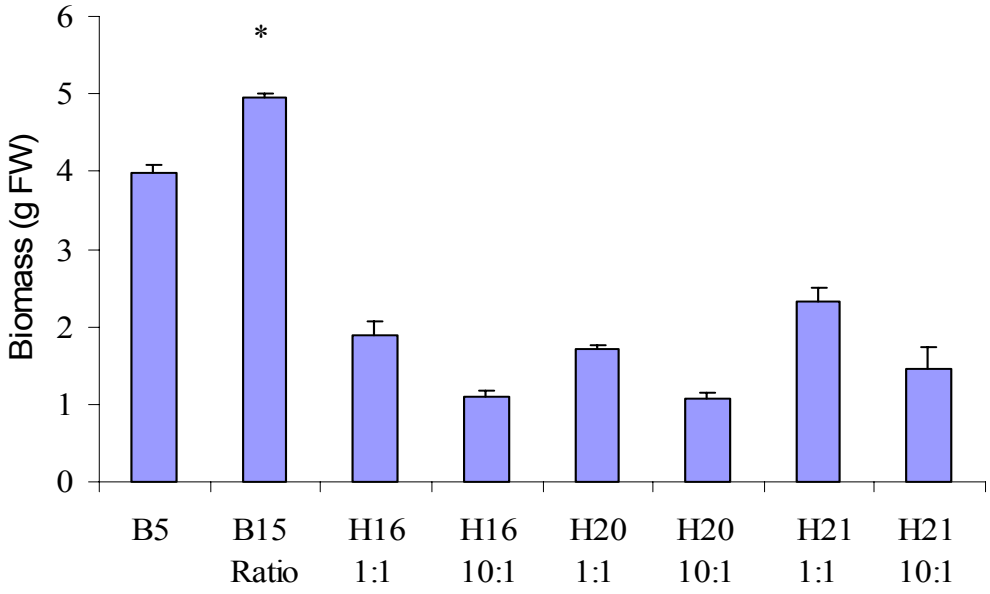

Figure 4.3. The effect on root growth of hormones added at different phytohormone-to-biomass ratios at day zero. Cultures were harvested at 14 days. Cultures were provided hormones either at the concentration shown in Table 1.1 or at one-tenth that level. The "*" represents the highest biomass value in the experiment.

4.1.6. Fresh Medium Effect on Growth

Experiments were run using a consistent, high amount of inoculum (5.0 grams) from 14-day-old cultures, in order to begin to determine if there was, indeed, a fresh medium influence on the amount of biomass produced in the presence of phytohormones as described in the previous experiments. Root growth was stimulated in all of the cultures by using fresh medium compared to cultures in Figure 4.4. Also, cultures supplied with ten times the original concentrations of H16, H20 or H21 showed no significant growth inhibition when compared to roots in which the original concentrations were used (Figure 4.5). Roots grown in the original concentrations of combination 16 produced, though barely, the highest levels

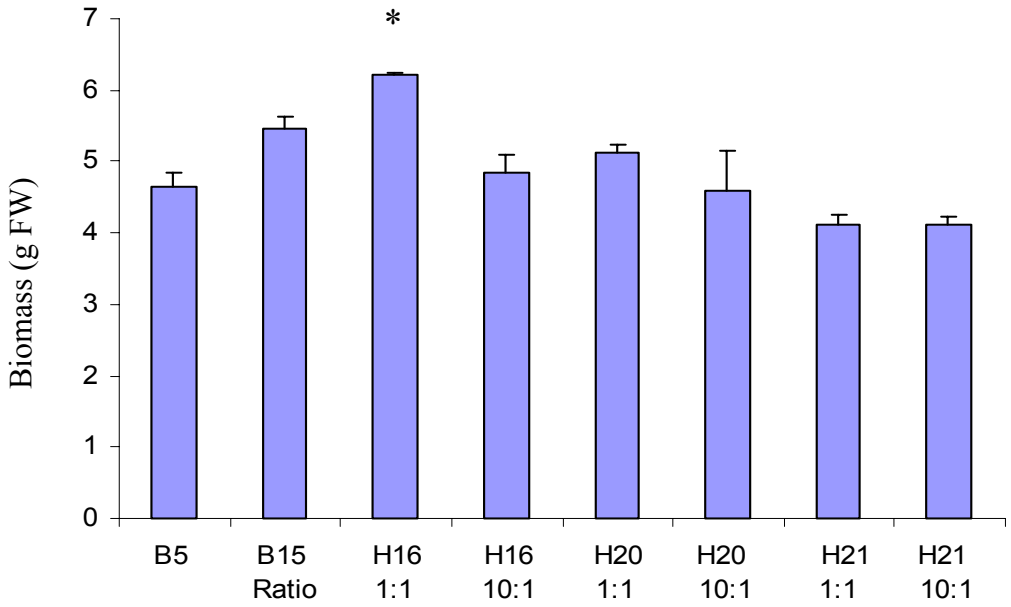

Figure 4.4. The effect of change in phytohormone-to-inoculum ratio added at day 14 on root growth of cultures harvested after 17 days of growth. Cultures were provided hormones either at the concentration shown in Table 1.1 or at 10 times that level. The "*" represents the highest biomass value in the experiment.

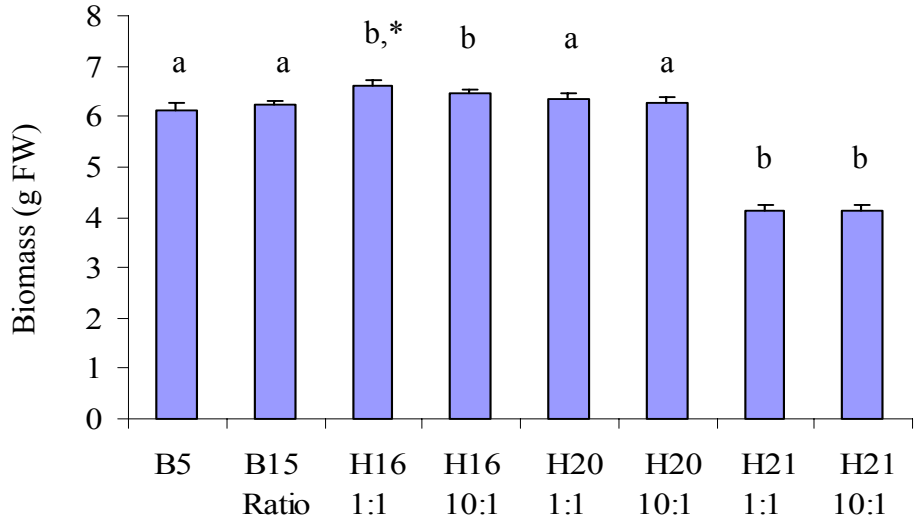

Figure 4.5 The effect on growth of hormones added at different phytohormone ratios and after provision of fresh medium to 5 grams of 14-day-old roots. Harvest was three days later. Cultures were provided hormones either at the concentration shown in Table 1.1 or at 10 times that level. The "*" represents the highest biomass value in the experiment. Letters (a and b) indicate a significance when comparing B15 medium controls, where "a" corresponds to the B15 controls.

of biomass. All the cultures seemed to produce comparable levels of biomass when compared to the medium B15 control, except for those roots cultured in both 1x and 10x concentrations of combination 21 where growth was still inhibited. It appears as if the addition of fresh medium to the culture obscures the ratio effect seen in Figures 4.3 and 4.4. This raises the question of whether the increases in biomass accumulation were the result of fresh medium addition, the manipulation of the culture (subculturing), or a combination of both.

4.1.7. Fresh Medium and Culture Manipulation Effects on Growth

To determine if there is both a fresh medium and a physical manipulation effect on artemisinin production and biomass accumulation, a series of experiments was run such that either old or fresh medium and teased or chopped roots were added at day 14 along with the optimum growth concentration for H16. Figure 4.6, shows that roots chopped and then grown in fresh medium do not significantly increase in mass unless hormones are also present (Figure 4.6). It is apparent that there is, indeed, a fresh medium and subculturing effect, albeit small, on the hairy roots of *A. annua.* While three days is a sufficient amount of time in which to quantitate a significant growth response, it is not clear whether not maximum growth occurs by three days post-hormone addition or later.

4.1.8. Determination of Peak Growth Post-Hormone Addition

In order to determine when the maximum biomass accumulation occurred post-hormone addition, roots were sampled, post-hormone addition, for six days. In the presence of hormone combination 16 and those grown without hormones, growth was linear for six

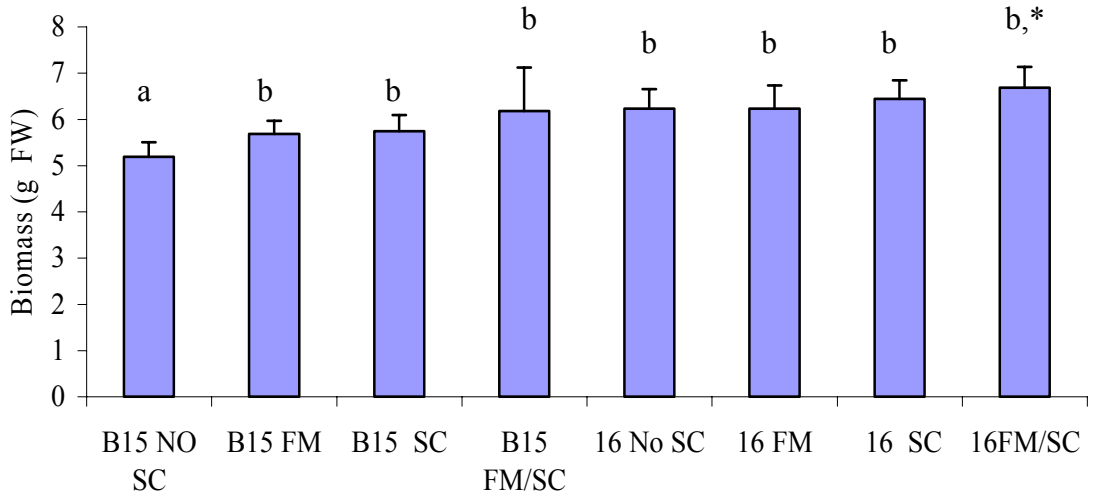

Figure 4.6. The effect on growth of hormones added at the original concentration of combination 16 at day 14 either in the presence or absence of fresh medium and or physical manipulation or both. FM, fresh medium added; SC, roots were chopped (physical manipulation). Cultures were harvested three days later. The "*" represents the highest value in the experiment. Letters "a" and "b" indicate significance when compared to B15 medium controls, where "a" corresponds to the B15 controls.

days (Figure 4.7). Thus, the maximum growth yield occurs later some where beyond three days post addition.

4.2. Individual Phytohormone Effects on Artemisinin Production

The individual affects of NAA, GA_3, ethephon, ABA, and BAP on hairy root development of *A. annua* have not been previously reported. Thus, it was useful to measure the effect that these phytohormones play on secondary metabolite production (i.e. artemisinin accumulation).

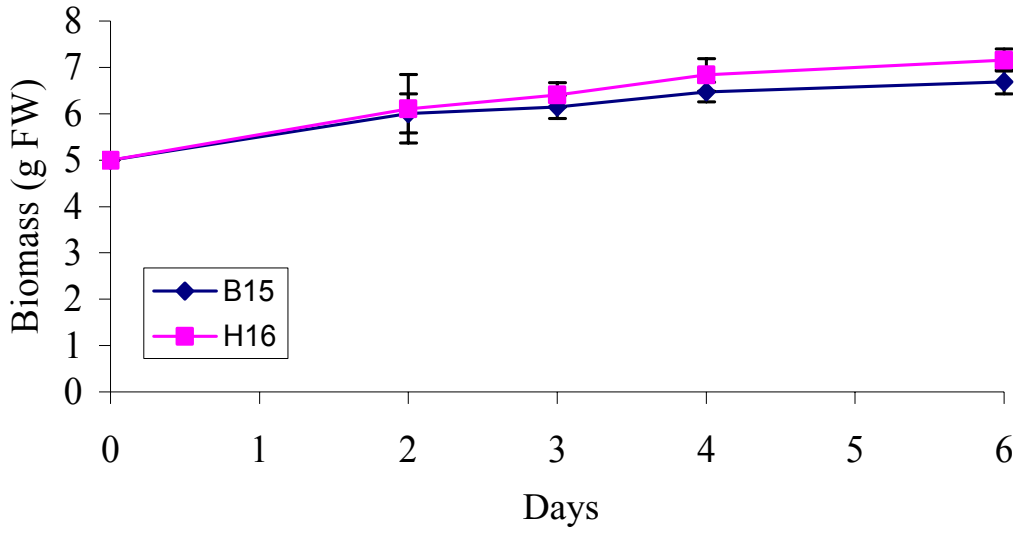

Figure 4.7 The effect on growth of hormones added at the original concentration of combination 16 at day 14 with fresh medium and physical manipulation. Cultures were harvested as shown above. The three day points were taken from Figure 4.6. All points represent the average of three samples. B15, no hormone addition; H16, hormone addition.

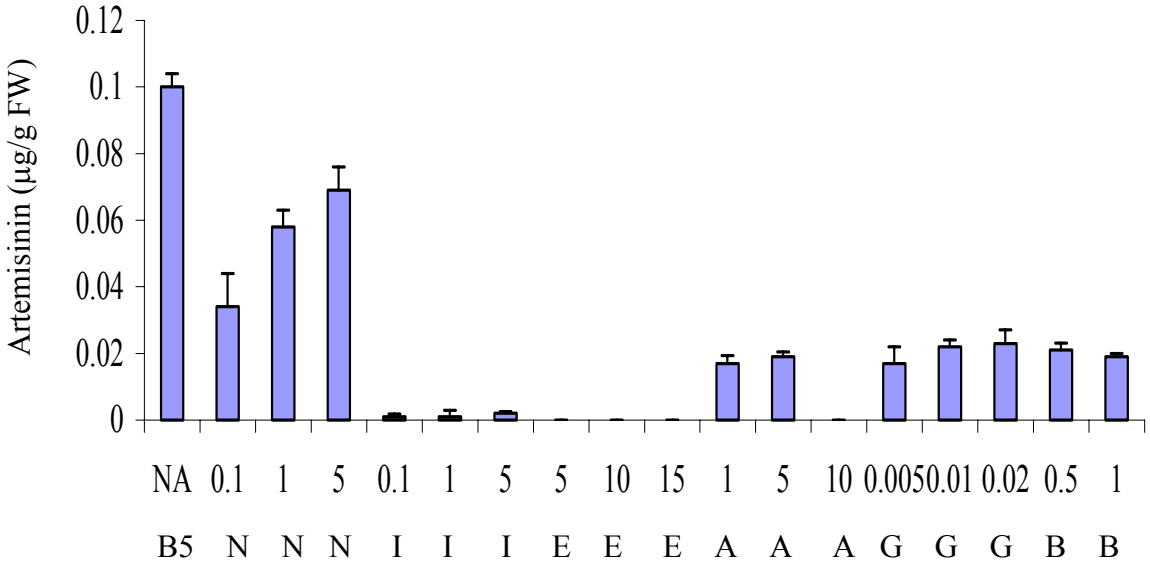

Figure 4.8 The effects of individual phytohormones added at day 0 on artemisinin production in cultures harvested after 14 days of growth. The "*", represents the highest overall value. N, NAA; I, IAA; E, ethephon; A, ABA; B, BAP; and B5, B5 control; NA, no addition. Concentrations on the x-axis represent mg/l quantities.

4.2.1. In Flasks: Bulk Artemisinin Production

Of the six individual hormones studied (Figure 4.8), it was observed that all concentrations of NAA produced the highest amounts of artemisinin, nearly 70 times higher than in cultures where artemisinin was barely detectable. Roots incubated in IAA yielded significantly less artemisinin than roots grown in NAA, at the same concentrations (Figure 4.8). These results show that providing cultures with the natural auxin, IAA, was more effective in inhibiting artemisinin accumulation than the synthetic NAA. Indeed IAA, ethephon, and ABA significantly inhibited artemisinin production compared to cultures fed 5.0 mg per liter NAA. Roots fed GA_3 and BAP produced considerably less artemisinin. No culture produced less artemisinin than those grown in ethephon. No disorganization of the root matrix was observed in any culture.

4.2.2. Two-Stage Application of a Four Hormone Combination: Affect on Artemisinin Production

In order to study the affects of medium composition and timing of a four-hormone treatment on artemisinin production, cultures were provided either with hormones at the day of inoculation or after 14 days of growth (Figure 3.1). When phytohormones were added at the day of inoculation, (Figure 4.9), artemisinin production in H16 was about three times that of roots grown in either H20 and H21 (Figure 4.9A)**.** However, roots grown in H16 produced 30% less artemisinin than roots grown in medium B15, and 65% less than in B5. Roots grown in both H20 and H21 yielded 25% the artemisinin produced in roots incubated in medium B15. Also, when hormones were added at inoculation, cultures grown in

medium B5 for 14 days produced roughly twice as much artemisinin than roots cultured in medium B15.

When roots were allowed to grow 14 days prior to phytohormone addition, they displayed a very different artemisinin production profile (Figure 4.9 B). Roots grown in H16 showed significant increases in specific artemisinin levels produced after 17 days of growth (Figure 4.9B) compared to their 14 day counterparts (Figure 4.9 A) and all other 17 day cultures (Figure 4.9 B). Also, after 17 days of growth, the cultures grown in medium B15 had greater specific artemisinin levels than those cultured in medium B5 (Figure 4.9 B), but less than their 14 day counterparts (Figure 4.9A).

Interestingly, when comparing the highest amount of artemisinin produced in cultures harvested at day 14 to the highest amount produced in those cultures harvested at day 17, the two-stage culture system seemed to provide no advantages in terms of increasing the specific artemisinin levels in roots (Figure 4.9A and B). Taking into account the biomass yield on a per liter basis, artemisinin production was not significantly different than levels in roots grown in combinations 16, 20 and 21, when hormones were supplied at inoculation, and was still considerably less than the B15 control. Even the B5 cultures produced more artemisinin (Figure 4.10 A). However, after 17 days of growth, the cultures fed hormones at day 14 provided a very different picture. Roots grown in H16 had total artemisinin yields significantly greater than B15, or B5 cultures and any of the other hormone combinations. However, total artemisinin levels still did not surpasses that of 14-day-old B5 cultures (Figure 4.10A and B).

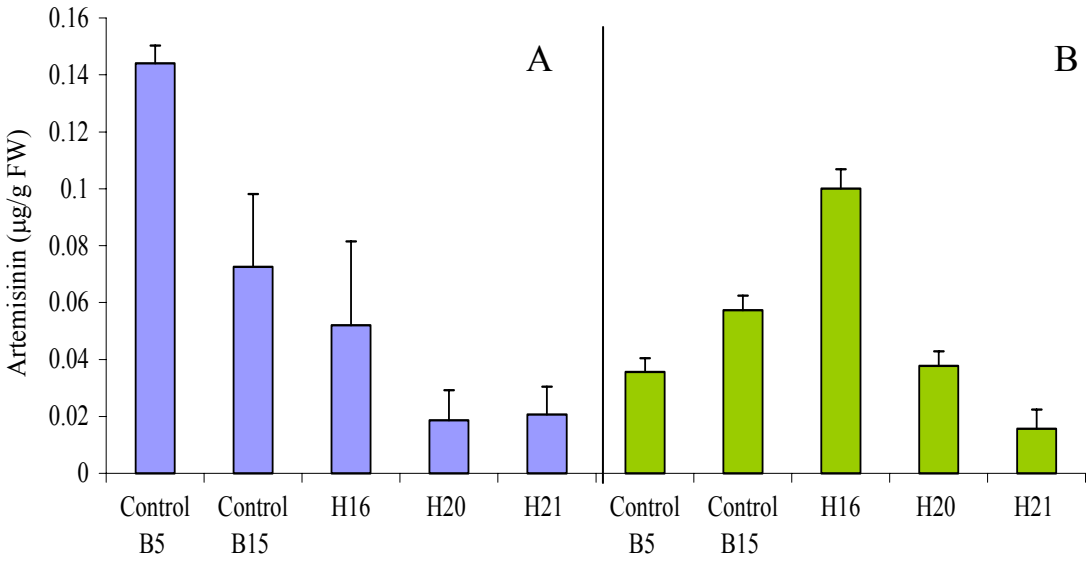

Figure 4.9 The affects of phytohormones on artemisinin production of hairy root cultures of *A. annua.* **A**, all cultures harvested after 14 days of growth; hormone cocktails No. 16, 20, and 21 were added at day zero to roots growing in B15 medium. **B,** all cultures were harvested after 17 days of growth; hormone cocktails No. 16, 20, and 21 were added at day 14 to roots growing in B15 medium and then were allowed the grow three days prior to harvest.

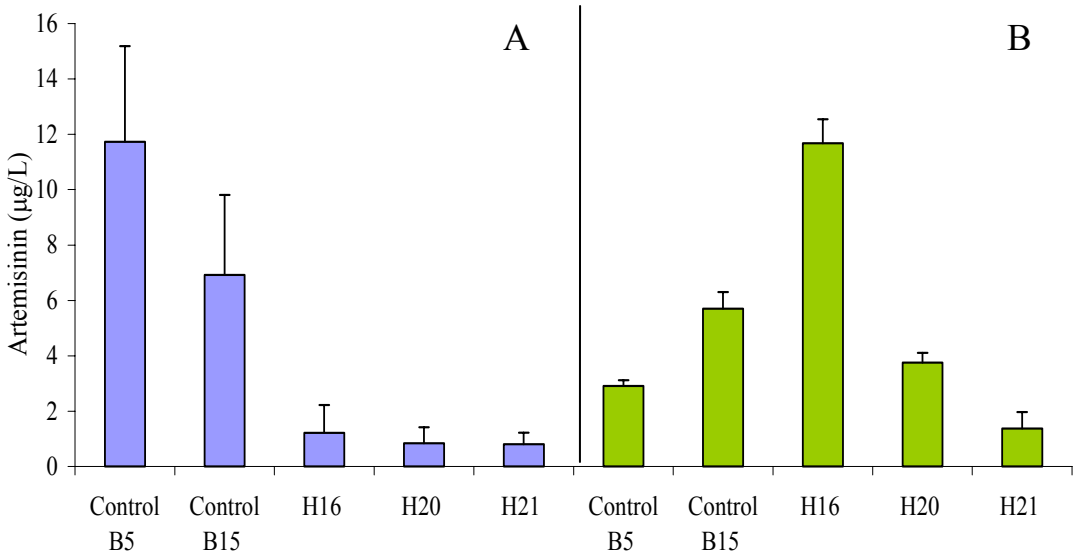

Figure 4.10 The overall affects of phytohormones on artemisinin production of hairy root cultures of *A. annua.* **A**, all cultures harvested after 14 days of growth; hormone cocktails No. 16, 20, and 21 were added at day zero to roots growing in B15 medium. **B,** all cultures were harvested after 17 days of growth; hormone cocktails No. 16, 20, and 21 were added at day 14 to roots growing in B15 medium and then were allowed the grow three days prior to harvest.

4.2.3. Effect of the Hormone-to-Biomass Ratio on Artemisinin Production

At inoculation, the ratio of hormones-to-biomass provided in the previous experiments was at a concentration about 10 times that provided to 14-day-old root cultures. The low levels of artemisinin observed in cultures fed hormones at day 0 might, therefore, be in response to the higher specific hormone concentration. Experiments were, therefore, conducted to test the effect of hormone-to-biomass ratio on root growth

To determine whether or not the ratio of hormone-to-root mass was critical to artemisinin production, cultures harvested at day 14 were supplied with the concentrations of H16, H20 or H21, either at the level shown in Table 1.1, or at one-tenth that level. Cultures harvested at day 17 were provided hormones at day 14 either at the level shown in Table 1.1 or 10 times that level (Figure 3.2). In this way each group of roots was provided hormones at a ratio of 1 to 10 of biomass.

Cultures supplied at day 0 with one-tenth the original hormone concentrations exhibited significant increases in artemisinin production when compared to roots in which the original concentrations were used (Figure 4.11). Roots cultured in one-tenth the concentrations of H16 and H20 displayed roughly twice the artemisinin levels of their 1x counterparts. However, roots cultured in H21 produced almost comparable levels of artemisinin at both hormone-to-biomass ratios (Figure 4.11).

When root cultures were fed the two ratios of hormones-to-biomass at day 14, similar responses were observed. Root cultures supplied with ten times the original concentrations showed significant artemisinin decreases when compared to roots grown in the original 1x concentrations (Figure 4.12). Furthermore, roots cultured in ten times the concentrations of H16, H20 and H21 produced as much as 50% less artemisinin than their 1x counterparts. In

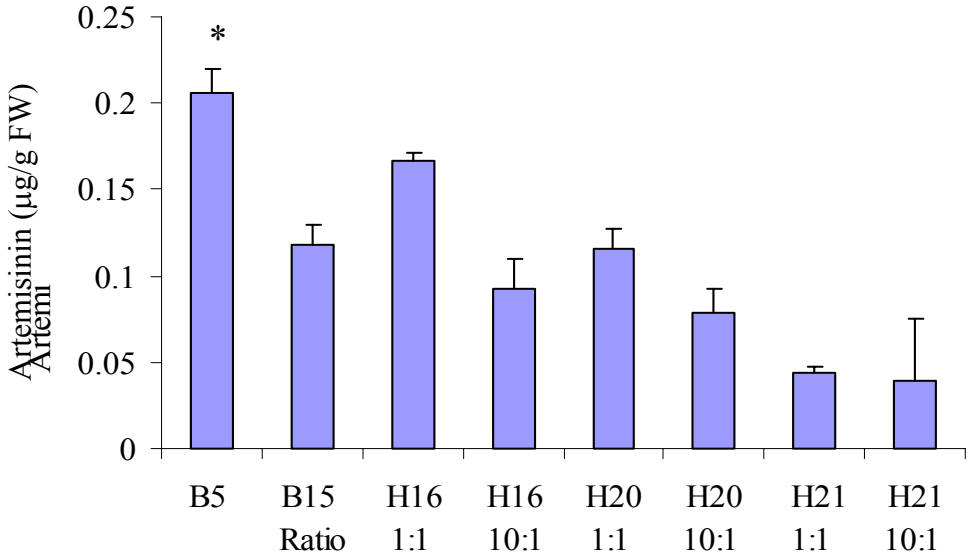

Figure 4.11. The effect on artemisinin production of hormones added at different phyto-hormone-to-biomass ratios at day zero. Cultures were harvested at day 14. Cultures were provided hormones either at the concentration shown in Table 1.1 or at one-tenth that level. The "*" represents the highest biomass value in the experiment.

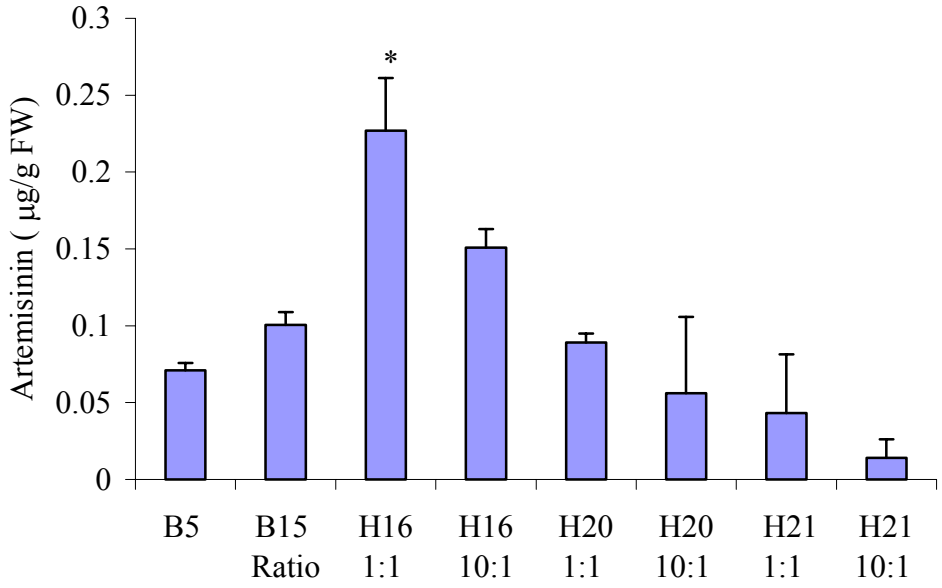

Figure 4.12. The effect on artemisinin production of hormones added at different phyto-hormone-to-biomass ratios at day 14. Cultures were harvested after three additional days of growth. Cultures were provided hormones either at the concentration shown in Table 1.1 or at 10 times that level. The "*" represents the highest artemisinin value in the experiment.

contrast, roots incubated in the original concentration of H16, had artemisinin levels greater than100 % above levels observed in B15 controls. Roots grown in both ratios of H20 and H21 produced lower artemisinin levels than those cultured in medium B15. Roots grown in both ratios of H16 yielded concentrations of artemisinin that were higher than those produced in roots cultured in medium B15 (Figure 4.12).

These data, in concert with the data in Figure 4.3, and 4.4, show that the hormone-to-biomass ratio has a strong influence on both growth and artemisinin production for roots inoculated at day zero and at day 14. Furthermore, three days appears to be a sufficient amount of time in order to quantitate root responses to phytohormone treatment, although it is not clear when artemisinin production and biomass accumulation are at optimum yield. The roots that were provided with hormones on day 14 were not subjected to the stress of culture manipulation, only the addition of fresh media.

4.2.4. Effects of High Inoculum levels and Fresh Medium on Artemisinin Production

Experiments were run using a consistent, high amount of inoculum (5.0 grams) from 14-day-old cultures in order to determine if there was an effect of fresh medium and physical manipulation on the amount of artemisinin produced in the cultures in the presence of two different levels of phytohormones (1x and 10x). After three days of incubation, roots were harvested from either 1x or 10x hormone concentrations H16, H20 or H21.

Figure 4.13 shows roots grown in fresh medium in the presence of phytohormones produced roughly twice as much artemisinin as roots not supplied with fresh medium, or roots grown in the presence of hormones without addition of fresh media (compare to Figure 4.11 and 4.12). The fact that only a doubling in artemisinin concentration was observed

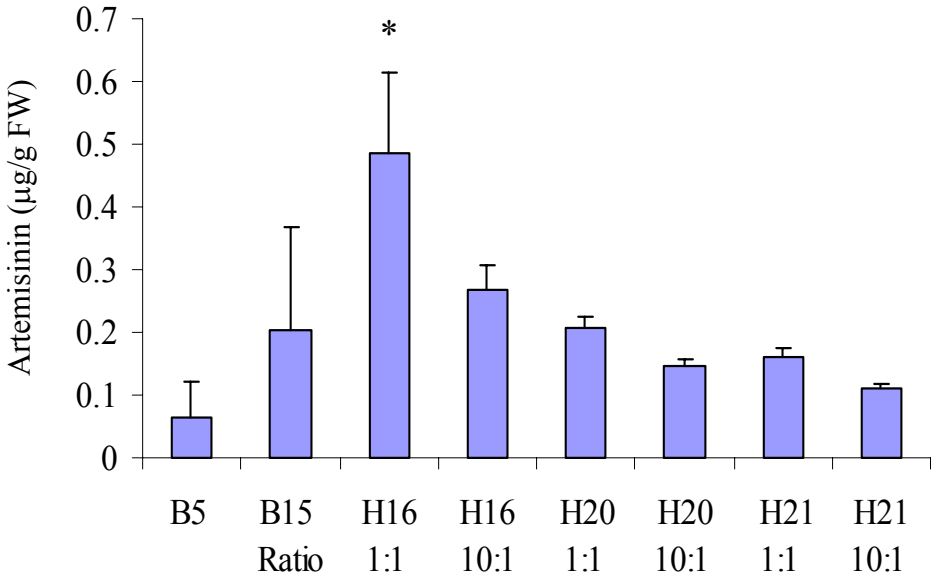

Figure 4.13. The effect on artemisinin production of hormones added at different phyto-hormone-to-biomass ratios and after provision of fresh medium to 5 grams of 14 day old roots. Harvest was three days later. Cultures were provided hormones either at the concentration shown in Table 1.1 or at 10 times that level. The "*" represents the highest value in the experiment.

when comparing roots subcultured into fresh medium to roots where fresh medium was not provided, suggests that the response was additive. Taken together, these data confirm two things: (1) there is a phytohormone-to-biomass ratio influence on artemisinin production, and (2) that the additions of fresh medium supplemented with hormones either stimulates terpenoid biosynthesis, or prevents the catabolism of the product, artemisinin. This raises the question of whether or not the increased artemisinin was the result of fresh medium addition, physical manipulation of the culture (chopping), or a combination of both.

4.2.5. Analysis of Fresh Medium and Manipulation Effects on Artemisinin Production

In order to better analyze fresh medium and physical manipulation effects on artemisinin production and biomass accumulation, a series of experiments was run in 2.8 L Fernbach flasks where either old or fresh medium, and/or teased (no chopping) or chopped roots were added at day 14 to the optimum growth phytohormone-to-biomass ratio for H16 (0.1x).

When fresh media was provided along with physical manipulation of the roots, the artemisinin levels were about two times as much as the artemisinin levels seen in roots where either one procedure or the other had been performed. This suggests that the addition of fresh medium together with subculture has an additive effect on artemisinin production (Figure 4.14). Furthermore, Figure 4.14 shows that in the presence both old medium and mock subculture (teased), hormones seem to play a critical role in stimulation of artemisinin production. There was roughly 100% increase in yield. Addition of fresh medium increased artemisinin levels above root cultures where no fresh medium had been added or no subculturing had taken place. When roots were only subcultured, the same effect was observed as was seen for those roots where only fresh medium and hormones were provided. There was a 100% increase in artemisinin as compared to the B15 controls (Figure 4.14)**.** Indeed it is apparent that there is a fresh medium and subculturing effect on the hairy roots of

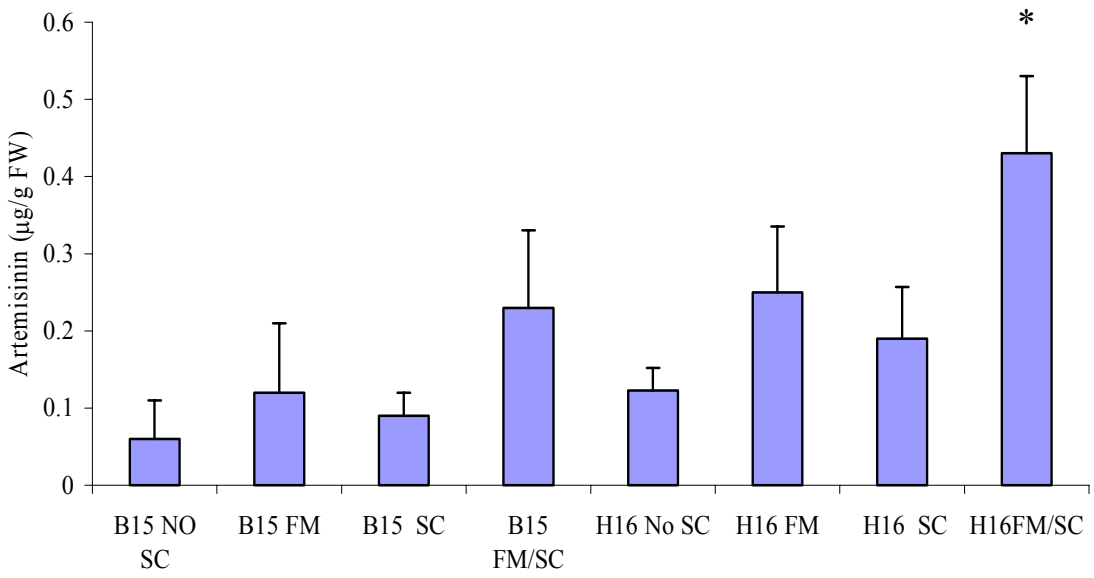

Figure 4.14. The effect on artemisinin production of hormones added at the original concentration of combination 16 at day 14 either in the presence or absence of fresh medium and or physical manipulation or both. Cultures were harvested three days later. The "*" represents the highest value in the experiment. FM, fresh medium addition; SC, physical manipulation.

A. annua. While it is apparent that three days is a sufficient amount of time in which to quantitate a significant artemisinin response, it is not clear whether not maximum production phosphate occurs three days post-hormone addition.

4.2.6. Determination of Peak Artemisinin Production Post-Hormone Addition

In order to determine if the maximum artemisinin production response occurs three days post-hormone addition, roots were assayed for artemisinin for six days post-hormone addition. Cultures that were grown in the presence of hormone combination 16 produced artemisinin almost linearly for six days, ultimately reaching about 1µg g^{-1} FW (Figure 4.15). In contrast, roots grown without hormones steadily declined in their artemisinin production

(Figure 4.15). Thus, maximum artemisinin production occurs more than three days post-hormone addition.

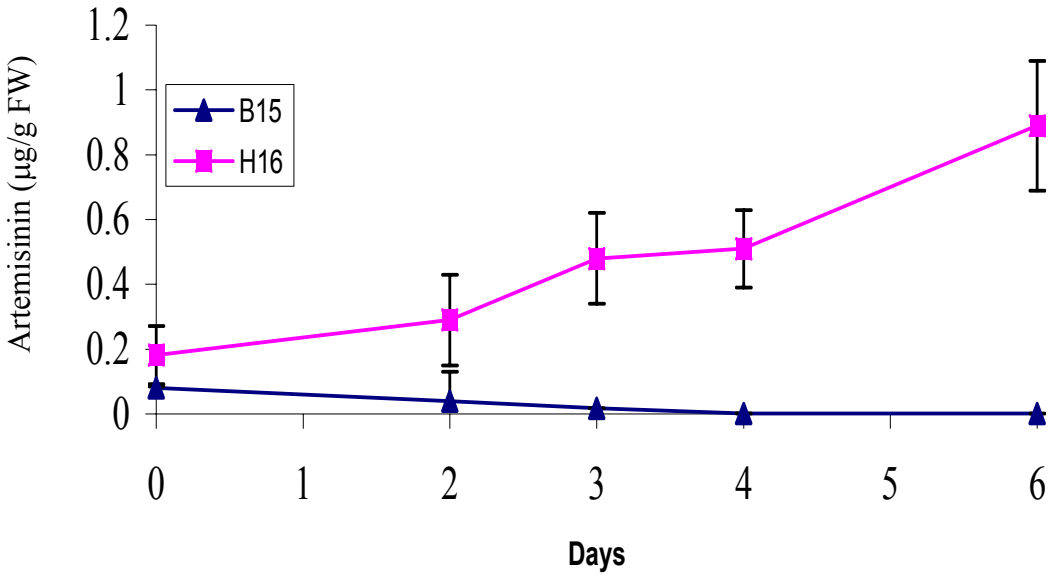

Figure 4.15. The effect on artemisinin production of hormones added at the original concentration of combination 16 at day 14 with fresh medium and physical manipulation. Cultures were harvested at two day intervals. The three day point was taken from Figure 4.12. All points represent the average of three samples. B15, no hormone addition; H16, hormone addition.

CHAPTER 5

DISCUSSION

5.0. Summary

The overall objective of this work was twofold: to increase fundamental understanding of the effects of exogenously applied phytohormones on growth and secondary metabolite production in hairy roots of *Artemisia annua,* and to develop an optimized two-stage culture system for artemisinin production. To my knowledge, this project may serve as the first definitive evidence demonstrating favorable responses to exogenous combinatorial hormone application with respect to root growth and secondary metabolite production by any hairy root species. Detailed analysis of artemisinin and biomass accumulation in *A. annua* hairy roots in the presence of phytohormones has uncovered effective individual phytohormone as well as mixed phytohormone concentrations suitable for single and bulk root growth, and artemisinin production. Also demonstrated is an effective phytohormone combination that allows for suitable growth and artemisinin production when used in conjunction with time of addition and provision of fresh nutrients and mechanical stress to the culture. While the findings in this report could still be optimized, they argue for the potential utility of a two-stage production scheme, incorporating combinatorial phytohormone use in concert with optimized growth medium to overproduce plant secondary metabolites *in vitro*.

5.1. Phytohormone Effects on Growth

Growth studies using polystyrene plates with single roots of *A. annua* established the dominance of the plant regulator gibberellic acid in many of the growth root characteristics

studied as well as in demonstrating the ability of the roots to produce higher amounts of root biomass in decreasing hormone environments (Table 4.1). Similarly, Ohkawa *et al.* (1989), Liu *et al.* (1997) and Bais *et al*. (2001) showed that GA stimulated growth of hairy roots. Furthermore, some studies showed that lower GA concentrations were more effective than higher ones (Bais *et al.* 2001; Ohkawa *et al.* 1989; Table 4.1).

Although auxins in the presence of sucrose are known to stimulate lateral branching (Rolland *et al.* 2002), *A. annua* hairy roots displayed similar branching patterns when grown either in IAA or GA as shown by their RGU values (Table 4.1) along with lower biomass accumulation in bulk cultures (Figure 4.1). Growth response to auxins is, however, not clearcut. For example, Luczkiewicz *et al.* (2002) stated that root biomass increased in the presence of auxin in hairy root cultures of *R. hirta* L., whereas, Lin *et al.* (2003) demonstrated that auxin has little influence on hairy root cultures of *L. flavum*. Considering that the transformation process with *A. rhizogenes* results in clones that are either super sensisitive to auxin or auxin overproducers (Giri and Narasu, 2000; Arroo *et al.* 1995), this likely explains the reported contrasting responses to auxin. Our clone YUT-16 is likely an auxin overproducer because bioassays have shown auxin is present in the culture medium (unpublished results). IAA is initially required to launch a population of quickly dividing pericycle cells that then form hormone-independent meristems (Laskowski *et al.* 1995), thus, alleviating the need for or an insensitivity to IAA (Reed *et al.* 1998). This may explain why *A. annua* root tips grown in IAA produced poor measurements in their number of laterals, length of the primary, lateral density, total length of laterals, average lateral length and total root length compared to tips grown in GA and even ABA (Table 4.1). At high concentrations IAA is also known to stimulate the production of ethylene (Crozier *et al.* 2000). Perhaps the

concentrations tested here were sufficient to stimulate ethylene production. This may explain why individual root tips grown in higher concentrations of both auxins grew poorly.

All root cultures grown in ABA produced high levels of bulk root biomass (Figure 4.1); although in single root studies GA was the most stimulatory (Table 4.1). Although ABA can inhibit shoot growth, it does not have a similar affect on roots or may even promote growth of roots (Crozier *et al.* 2000). This may explain the increased growth seen in bulk cultures provided with ABA, but does not explain the single root results.

Consistent with my results Sauerwein *et al.* (1992), Dhingra *et al.* (2000), and Luo *et al.* (2001), reported that the growth rate of transformed hairy roots with *A. rhizogenes* increased in the presence of low concentrations of cytokinins, auxin, and gibberllins. Indeed, maximum biomass was obtained in roots cultured in the lowest concentrations of most of the phytohormones tested (Table 4.1 and Figure 4.1).

Cytokinins stimulate cell division and shoot morphogenesis (Crozier *et al.* 2000). Consistent with prior observations (Bunk, 1997), single and bulk roots grown in exogenously applied cytokinin (BAP) showed little lateral root initiation. Interestingly, addition of BAP at these concentrations did not promote root elongation (Table 4.1). Bais *et al,* (2001), reported that increasing the cytokinin: auxin ratio in the hairy roots of *C. intybus* resulted in decreased biomass. There may have been a similar interplay between exogenously fed cytokinin and the endogenous auxin found in this clone of *A. annua* that is responsible for the decreased growth seen in cultures fed BAP.

Single and bulk roots cultured in all concentrations of ethylene yielded the worst growth measurements (Table 4.1, Figure 4.1). Although exogenous addition of ethylene (10 mg/l) to the culture medium stimulated growth of A. *annua* L. plantlet cultures, there appears

to be no report on its effects on roots (Fulzele *et al.* 1995). It appears, therefore, that based on my study, ethylene inhibits *A. annua* hairy root growth.

Time of addition of phytohormones plays a crucial role in growth both in single and bulk root responses (Table 4.2 and Figure 4.2, respectively) Addition of phytohormones H16, H20 and H21, at day zero, inhibited growth compared to the medium B5 controls. Addition of hormones (14 days) later in the growth cycle decreased the adverse root growth effects and stimulated growth in *A. annua* cultures, especially in H16 (Table 4.2 and Figure 4.2). These results suggested that the specific concentration of hormones ($\mu g\ L^{-1}\ g^{-1}$ FW) was crucial as discussed in Section 5.4.

5.2. Phytohormone Effects on Artemisinin Production.

Unlike root growth, artemisinin content increased with increasing phytohormone concentration in cultures provided with NAA, IAA, ABA and GA_3 (Figure 4.8). In contrast, cultures grown in increasing amounts of ethephon and BAP, resulted in decreased amounts of artemisinin (Figure 4.8).

Sa *et al*, (2001), reported that increases in endogenous (iPA and PA) levels of cytokinin increases artemisinin content 70 % over controls. Apparently exogenous application of the synthetic cytokinins, BAP, used in the present studies is not as effective as increasing endogenous cytokinins via (*ipt*) gene transfer. However, Sa and his group (2000) used transformed shoot cultures where cytokinin : tissue ratios are high (Crozier *et al.* 2000**)**. In contrast, Bais *et al*, (2001), also reported that increasing the cytokinin : auxin ratio in hairy root cultures resulted in decreased secondary metabolite yields *in vitro*. Furthermore, the critical concentration of cytokinins required for artemisinin biosynthesis is not known.

The exogenous application of phytohormones to transformed root cultures has been shown in some cases to result in the loss of root integrity and secondary metabolite production (Rhodes *et al.* 1994, Dinghra, 2000). Artemisinin content in was lowered in the presence of phytohormones compare to the hormone-free control flasks (Figures 4.8 and 4.9) suggesting that these hormones are in some way inhibitory to artemisinin production in hairy roots of *A. annua* clone YUT-16. However, on a per liter basis, cultures grown in H16 produced artemisinin levels similar to the B5 control flasks (Figure 4.10).

Application of ethylene to *A. annua* hairy roots produced the lowest artemisinin accumulation, suggesting that terpenoid accumulation is inhibited by ethylene. Roots cultured in NAA produced the highest levels of artemisinin suggesting that it is less inhibitory to production than the other phytohormones. Similarly, addition of indole-3-butyric acid (IBA) and NAA stimulated ajmalicine and ajmaline production compared to *R. micrantha* hairy roots cultured in hormone-free medium (Sudha *et al.* 2003). Also, coniferin production in *L. flavum* was significantly increased in the presence of auxin. Earlier work by Bunk (1997) suggested that in the presence of high levels of auxin, artemisinic compound yield is increased in *A. annua* hairy roots compared to hormone-free controls (data not shown). This is consistent with my findings.

Time of addition of phytohormones plays a crucial role in artemisinin production in bulk roots (Figure 4.9). Addition of phytohormone combinations H16, H20 and H21, at day zero, inhibited both artemisinin production and growth compared to the controls. Luo *et al.* (2001) also observed boosts in paclitaxel accumulation roughly 5 times that of the controls with the addition of phytohormones after 12 days growth of *T. chinensus* suspension cultures.

It appears that both *Taxus* and *Artemisia* may have similar time dependent processes that respond to the presence of phytohormones.

Artemisinin levels are stimulated by combination H16 (Figures 4.9 and 4.10). Given the limited literature on combinatorial phytohormone treatments, I can only speculate that there might be less antagonistic hormone interplay with combination H16 than with either H20 or H21 as H16 resulted in overall artemisinin levels above both controls and levels similar to the 14 day medium B5 controls. It is possible that roots grown in H20 and H21 may require longer periods of incubation in order to observe a marked effect on either growth or artemisinin production.

5.3. Medium Effects on Growth and Artemisinin Production

B15 medium composition can either inhibit or stimulate root growth and artemisinin production depending upon inoculum size and length of culture (Tables 4.2 and 4.3 and Figures 4.2 and 4.9). Growth increases in B15 medium compared to that in B5 are likely due to a higher amount of available carbon in the medium B15, 5% sucrose (Appendix A1). The extra carbon appears to influence artemisinin production, but only after cultures increase their biomass (Figure 4.9). Growth and development is governed by regulators and environmental cues that are themselves modulated and coordinated by the available carbon source (Koch, 1996; Sheen *et al.* 1999; Smeekens, 2000). A wide variety of genes are known to be sugar regulated at the transcriptional level, including genes involved in photosynthesis, carbon and nitrogen metabolism, response to stress, and secondary metabolism in different plant species (Rolland *et al.* 2002; Sheen *et al*. 1999; Smeekens, 2000). Consequently it is not unreasonable to consider that the extra carbon may be up or

down regulating transcription events late in growth (Figure 4.9) in the hairy roots of *A. annua* that led to the production of higher level of artemisinin.

5.4 Hormone-to-Biomass Ratio Effects on Growth and Secondary Metabolism

5.4.1. Growth

In cultures with phytohormones provided at inoculation with one-tenth the original hormone concentrations, growth increased significantly compared to roots in which the original concentrations were used (Figure 4.3). Roots cultured in one-tenth the concentration of H16, H20 and H21 displayed about a two-fold increase in biomass accumulation compared to their 1x counterparts. Figure 4.3 also shows the B15 control roots outperform either experimental condition (Figure 4.3)**.** It became apparent that not only the amount of biomass present at the time of phytohormone addition but also the amount of phytohormone added controls root development (Figure 4.3). When 14-day-old root cultures were supplied with ten times the original phytohormone concentrations used in experiments shown in Table 1.1, growth was also inhibited compared to roots that were fed the original concentrations (Figure 4.4). Consistent with Smith's data (Table 1.1), *A. annua* hairy root growth is suppressed upon addition of phytohomones, however, interestingly, when lower amount of phytohormones are present in the medium, the adverse growth effects are diluted. Inhibition of growth was particularly high for roots grown in H16 10x. Conversely, roots grown in H21 showed no growth inhibition after the higher 10x concentration. Most notable is the result showing that roots incubated with 1 x of H16, stimulated root biomass accumulation 12 percent above the medium B15 controls (Figure 4.4). Taken together these results show that there is, indeed, a significant ratio effect of hormones on growth.

In concert with previous data, more root growth should have occurred at lower hormone-to-biomass ratios than the higher ones. Roots cultured in H16 produced the highest levels of biomass when harvested after three days post inoculation. Again, hormone interplay may be less antagonistic in H16 than in either H20 or H21. Another useful outcome was that three days was sufficient time to observe a hormone-dependent growth response for all combinations studied except in H21. This suggests that the phytohormone concentrations in either H20 or H21 may be too weak to promote high levels of biomass. (Figures 4.3 and 4.4). It is clear that the phytohormone-to-root biomass ratio is crucial to growth of these cultures.

Upon adding fresh nutrients in the form of adding fresh medium to cultures, both root growth and artemisinin levels were stimulated (Figures 4.5 and 4.13, respectively). Carbon status has been shown to regulate growth and development (Koch, 1996; Sheen *et al.* 1999; Smeekens, 2000). The replenished carbon source may set in motion a cascade of events that ultimately result in better growth and sugar regulated transcription events (Rolland *et al.* 2002) that yield higher amounts of artemisinin. Again, roots cultured in fresh medium in the presence of H16 produced the highest artemisinin yields. The provision of fresh medium had an adverse affect on roots grown in the presence of H21, as evidenced by the relatively low amount of biomass. This suggests that the concentrations of phytohormones found in H21 inhibit growth regardless of the ratio. All the data suggest that the provision of fresh nutrients stimulates both growth and artemisinin production. It also became apparent that only the data from roots grown in the presence of H16 should be explored further.

5.4.2 Artemisinin Production

A similar response to that observed for growth was also found for artemisinin production. Artemisinin yields were higher when the hormone to biomass ratio was optimal. (Figures 4.11, and 4.12). For example, the addition of phytohormones at day 0 at one-tenth the original hormone concentrations, significantly increased artemisinin production when compared to roots in which the original concentrations were used (Figure 4.11). Also, roots cultured in one-tenth the concentrations of H16 and H20 displayed a two-fold increase in artemisinin. Curiously, roots cultured in H21 produced almost comparable levels of artemisinin at both hormone-to-biomass ratios (Figure 4.11) It would seem that here the ratio does not matter. Or perhaps that H21 is not stimulatory to artemisinin production when considering the fact that it failed to elicit a favorable response in previous experiments (Figure 4.11).

Roots fed the two ratios of hormones-to-biomass at day 14, showed similar responses. Consistent with data in Figure 4.11, roots given higher concentrations of phytohormones, ten times the original concentrations, displayed significant artemisinin decreases when compared to roots grown in the original 1x concentrations (Figure 4.12). Moreover, roots cultured in ten time the concentrations of H16, H20 and H21 produced about half the artemisinin their 1x counterparts did. In contrast, roots incubated in the original concentration of H16, had artemisinin levels double that observed in B15 controls. Also H20 and H21 produced low levels of artemisinin. All the evidence seems to suggest that H20 and H21 may not be stimulatory to artemisinin production under these conditions. Roots grown in both ratios of combination 16 yielded concentrations of artemisinin that were higher than those produced in roots cultured in medium B15 (Figure 4.12). Still roots grown in medium B5 produced as

much artemisinin as did the roots grown in H16 (Figures 4.11 and 4.12). It was evident that inoculum size coupled with lower biomass to hormone ratios did not significantly increase artemisinin yields beyond B5 controls.

In concert with the data in Figure 4.3, and 4.4, these data show that the hormone-to-biomass ratio has a strong influence on both growth and artemisinin production for roots inoculated at day zero and at day 14. The roots that were provided with hormones on day 14 were not subjected to the stress of culture manipulation, only the addition of fresh media.

5.5 Mechanical Manipulation and Subculture Effects

5.5.1 Growth

Upon adding fresh medium to cultures, root growth was stimulated in cultures grown in H16 and H20 (compare Figure 4.5 and 4.4) with, roots cultured in fresh medium in the presence of H16 producing the highest levels of biomass (Figure 4.5). Addition of fresh nutrients had no effect, stimulatory or inhibitory, on root growth in H21 cultures. Perhaps, three days post inoculation, the roots may be in a lag phase trying to adjust to the phytohormones. All cultures fed phytohormones H16, H20 and H21 produced dense callus tissue suspension cells to some degree. While disorganization of the root matrix was not a characteristic studied, this parameter may explain artemisinin and growth decreases. Bais *et al.,* (2001) showed that the hairy roots of *C. intybus* in the presense of cytokinins and auxins, rapidly undergo disorganization of the root matrix ultimately yielding suspension cultures. While obvious in cultures fed phytohormones at day 0, the appearance of free cells in the surrounding medium was not as evident in cultures provided phytohormones at day 14. Perhaps the degree of this phyto-physiological occurrence relative to the amount of

phytohormone exposure (i.e. time of addition) may explain variances in root growth in *A. annua* hairy root cultures.

Also, while roots fed 1x H16 and fresh medium significantly increased biomass levels over the B5 and B15 controls, the hormone-to-biomass ratio effect evident in earlier experiments (Figures 4.3 and 4.4) was heavily diluted (Figure 4.5). There appeared to be no statistical difference between roots fed either 1x or 10x the concentrations in H16. It may be that all the roots were experiencing a lag in growth, and the phytohormones in H16 are facilitating faster recovery from the stress of subculture (Figure 4.5).

Interestingly, in Figure 4.6, when roots were chopped and then grown in fresh medium, they did not significantly increase in mass unless hormones are also present (Figure 4.6). When analyzing the results of mechanical manipulation (i.e. subculture, chopping or transferring) of the root cultures in concert with the provision of fresh medium, the combination of these techniques stimulated root growth above either lone procedure (Figure 4.6). The lowest levels of biomass occurred in the absence of subculture and fresh medium. Surprisingly, chopping of the roots did not effect the roots adversely. According to previous work in our lab (data not shown), *A. annua* hairy roots undergo a lag period following subculture. Perhaps the addition of fresh medium and the phytohormones concentrations in H16 together facilitate exponential growth regardless of wounding. There seems to be a fresh medium effect on growth.

5.5.2 Artemisinin Production

When fresh medium was added to the dense root cultures, artemisinin levels were stimulated (Figures 4.13). Roots cultured in fresh medium in the presence of H16 produced

the highest artemisinin yields overall. Interestingly, roots fed H16 1x and fresh medium significantly increased biomass levels over the B5 and B15 controls as well as roots fed H20 and H21. The hormone-to-biomass ratio effect evident in earlier experiments (Figures 4.11 and 4.12) remained consistent.

Mechanical manipulation (i.e. subculture, chopping or transferring) of the root cultures stimulated artemisinin production (Figure 4.14). The lowest levels of artemisinin production occurred in the absence of both subculture and fresh medium. Further, when cultures were both subcultured and provided with fresh medium along with H16, artemisinin levels were stimulate to the highest levels (Figure 4.14). The increased stimulation of artemisinin with fresh medium and then mechanical manipulation is additive (Figure 4.14).

ABA induces gene transcription (Crozier *et al.* 2000) in response to wounding. Perhaps in cultures fed H16, the concentrations of phytohormones favorably interact with endogenous hormone levels, potentially intiatiating a similar mechanism of ABA induced gene transcription, that results in more artemisinin production. Or perhaps there exists a more efficient sugar regulated pathway for the production of secondary metabolites, specifically artemisinin in hairy roots of *A. annua*. Alternatively, the production or lack thereof the suspension cells and callus tissues in these cultures may also be contributing factors. After 14 days *A, annua* hairy roots begin to form callus tissue regardless of hormone addition. Since secondary products are typically produced in differentiated tissues conversion of these tissues to callus could result in diminished artemisinin production.. If the phytohormone concentrations in H16 inhibit callus formation in the roots, this may explain why roots fed H16 in Figure 4.14 produced higher levels of artemisinin compared to B15 controls.

5.6. Determination of Conditions for Maximum Biomass and Artemisinin Production

The length of phytohormone incubation time required for maximum growth and artemisinin production using H16 coupled with provision of fresh medium and mechanical manipulation was not determined (Figures 4.7 and 4.15). However, at six days post-inoculation both root growth and artemisinin levels seemed to be steadily increasing. Due to time constraints, analysis longer than six days were not run. It is clear, however, that both growth and artemisinin production peak somewhere beyond three days post inoculation.

CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

6.1 Summary and Conclusions

In summary, herein lies the first experimental evidence that hairy roots of *A. annua* can produce elevated biomass and artemisinin levels using a two-stage culture system incorporating the use of multiple phytohormones (e.g. NAA, GA3, Ethephon, and ABA) in combination with subculturing methods. Single and bulk root growth responded best to decreasing hormone concentrations. However, in terms of artemisinin roots grown in gibberellic acid, ABA and IAA produced the highest root growth characteristic values. Roots supplied with the synthetic auxin, not only outperformed natural IAA, but all other phytohormones in this study. Roots supplied with ABA produced the highest levels of root biomass. Time of addition played an important role in root biomass accumulation and artemisinin yield. There seemed be a hormone-to-biomass ratio effect, suggesting an optimal range of specific hormone concentrations favorable to both growth and artemisinin production. Also, the provision of fresh medium and physical manipulation stimulated both root growth and artemisinin production. Fresh medium and physical manipulation seemed to act additively in stimulating both growth and artemisinin production. Lastly, maximum artemisinin production and root growth occurs sometime beyond three days incubation in H16 medium.

6.2 Future Research

Future work involving this production scheme might involve studies to uncover the peak of artemisinin production as well as to define critical concentration ranges for the maximum root biomass as well as artemisinin yield via another fractional factorial analysis. It would also be useful to run this two-stage process using the individual hormones. Pronounced effects on growth and artemisinin production over the hormone-free controls and the combinatorial hormone data would suggest hormone-hormone antagonism. These studies would allow for the rapid and effective process design for future experiments.

Also, Sa *et al.* (2000) observed increased endogenous levels of the cytokinins isopentenyl adenine (iPA), not BAP. Further experiments using natural exogenous cytokinins that are found in *A. annua,* especially iPA, should be run before one can preclude the effects of cytokinins on hairy roots of *A. annua*.

Disorganization index studies should also be run on each culture provided with phytohormones. The level of disorganization in each combination may begin to explain the differences observed between H16, H20 and H21 with respect to growth and artemisinin production.

Lastly, experiments using brassinosteroids might prove useful considering that Wang *et al.* (2003) demonstrated a 57% increase in artemisin yields over brassinosteroid-free cultures of *A. annua* hairy roots, in the presence a synthetic steroidal lactone, (22S,23S)-homobrassinolide (SSHB).

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APPENDIX A1

B5 Stock Solution Preparation: **B15 Modification**

Stock Name	Concentration	Use:	Use:
1. 1.01 M $(NH_4)_2SO_4$	134 g/l	1ml/l	1ml/l
2. 0.048 M H_3BO_3	3 g/l	1ml/l	1ml/l
3. 1.02 M $CaCl_2 \cdot 2H_2O$	150 g/l	1ml/l	1ml/l
4. 0.1 mM $CoCl_2 \cdot 6H_2O$	0.025 g/l	1ml/l	1ml/l
5. 0.1 mM $CuSO_4 \cdot 5H_2O$	0.025 g/l	1ml/l	1ml/l
6. 1.01 M $MgSO_4 \cdot 7H_2O$	250 g/l	1ml/l	1ml/l
7. 0.059 $MnSO_4 \cdot H_2O$	10 g/l	1ml/l	1ml/l
8. 4.5 mM KI	0.750 g/l	1ml/l	1ml/l
9. 0.11 M Na_2EDTA	37.3 g/l	1ml/l	1ml/l
10. 1.03 mM$Na_2MoO_4 \cdot 2H_2O$	0.25 g/l	1ml/l	1ml/l
11. 1.08 M $NaH_2PO_4 \cdot 2H_2O$	150 g/l	1ml/l	0.924ml/l
12. 6.9 mM $ZnSO_4 \cdot 7H_2O$	2 g/l	1ml/l	1ml/l
13. 0.055 M I-Inositol	9.91 g/l	10ml/l	10ml/l
14. 0.03 Thiamine	10 g/l	1ml/l	1ml/l
15. 4.86 mM Pyridoxine	1 g/l	1ml/1	1ml/l
16. 8 mM Nicotinic Acid	1 g/l	1ml/l	1ml/l
17. 0.1 $FeSO_4 \cdot 7H_2O$	27.8 g/l	1ml/l	1ml/l
18. 2.47 M KNO_3	249.7 g/l	10ml/l	6ml/l
Surcose:		30 g/l	50g/l
pH:		5.7	5.7

Modifications in inorganic phosphate and nitrogen content as well as the sucrose level are taken from Hemmavahn, (1995).

APPENDIX A2

Sterilization Procedure for six-well plates:

For: Multiwell ™ Primaria ™ 6 well plates, Becton Dickinson ®.

1. Plates can be thoroughly washed with soap (7x brand) and water (Wash each individual well and the cover. Rinse thoroughly.
2. Air dry.
3. Once dry, the plates can be modestly rinsed with 70% ethanol only, never acetone.
4. Air dry with lid securely on in a hood
5. Microwave for and not exceed 3* minutes on high heat in a commercial microwave; microwave plates individually, for best results, with lid in place.

Note: *The plate is now sterile for up to one day if sterile environment is not disturbed.*

* When heating several plates exercise caution. Energy trapped in the microwave from consistent use can cause losses in plate structural integrity. This also occurs after 3 minutes has elapsed. To avoid structural damage allow two minutes between each sterilization attempt. Extreme plate temperatures are not required for sterilization, thus one should be able to handle the plate after each attempt.